Strategies to reduce the impact of opioid-induced adverse effects

by

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Declaration of originality

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The research associated with this thesis abides by the international and Australian codes on animal experimentation, the guidelines by the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. All animal experiments were approved by the Animal Ethics Committee (AEC), University of Tasmania, Australia (Animal Ethics approval number: A0013864).

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List of publications and statement of co-authorship

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We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published or under review peer-reviewed manuscripts contributing to this thesis.

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List of abbreviations

'~'	an approximate value
'-'	pre-administration (time-point)
μM	micromolar
0 min	basal or before treatment
3D	three dimensional
5-HT	serotonin (receptor)
AAW	acetic acid writhing test
AC	adenylyl cyclase
ADME	absorption, distribution, metabolism and excretion
AQP3	aquaporin-3
ATP	adenosine triphosphate
AUC	area under the curve
βarr	β-arrestin
βarr1	β-arrestin-1
βarr2	β-arrestin-2
β-FNA	β-funaltrexamine
b.i.d.	two times daily
BBE	blood brain barrier
BL	baseline latency
BMI	body mass index
BPS	British Pharmacological Society
BWT	body-weight

Bzl	Benzyl
cAMP	cyclic adenosine monophosphate
CL	cut-off latency
CPIP	Chronic Postischemia Pain
CRE	cAMP response element
CREB	cAMP response element binding protein
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
CYP	Cytochrome P450 enzyme
cdk5	cyclin-dependent kinase 5
D1	dopamine receptor-1
D2	dopamine receptor-2
DA	dopamine neurons
DARPP-32	dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DALDA	H-Tyr-D -Arg-Phe-Lys-NH_2
DAMGO	[D-Ala2, N-MePhe4, Gly-ol]-enkephalin
DM	diabetes mellitus
DMSO	dimethylsulphoxide
Dmt	2′, 6′-dimethyl-l-tyrosine
dns	does not stimulate (a receptor)
DOP	delta-opioid (δ-opioid receptor)
DPDPE	[D-Pen2,D-Pen5]-enkephalin
DPN	diabetic peripheral neuropathy
DRM	dermorphin
EC_{50}	effective dose 50%,

Emax	maximum effect in percentage
FEN	fentanyl
Fig.	Figure
g	gram
GAB	gabapentin
GABA	gama-aminobutyric acid
GI	gastro-intestinal
Gly	Glycine
GlyRα3	cAMP and dephosphorylate glycine receptor type α3
GPCR	G-protein-coupled receptor
GPI	guinea pig ileum
GTPγ^{35}S	Guanosine-5'-O-(3-[^{35}S]thio)triphosphate
h	hour
HCl	hydrochloric acid
HP	hot plate test
HPL	hot plate latency
i.c.v.	intracerebroventricular
IGF-1	insulin-like growth factor 1
i.p.	intraperitoneal
i.t.	intrathecal
i.v.	intravenous
IASP	International Association for the Study of Pain

IUPHAR	International Union of Basic and Clinical Pharmacology
K_e	antagonistic efficacy
K_i	affinity value
KO	knock-out
KOP	kappa-opioid (κ- opioid receptor)
L	litre
m	metre
M.HCl	morphine hydrochloride
MCS	Multi-Conditioning System
MDV	mouse vas difference
mg	milligram
min	minutes
mM	milimolar
MOP	mu-opioid (μ-opioid receptor)
MOR	morphine
MPE	Maximum Possible Effect
MS	morphine sulphate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (assay)
n/a	not applicable / available
NAc	nucleus accumbens
nBNI	norBNI
nd	not detected
NK_1	Neurokinin 1 (receptor)

NLX	naloxone
nM	nanomolar
NMDA	N-methyl-D-aspartate (receptor)
NOP	nociceptin/orphanin-FQ (non-opioid receptor)
norBNI	norbinaltorphimine
NTL	naltrindole
NTX	naltrexone
OF	open-field test
OL	opioid ligand
OR	opioid receptor
os.pump	osmotic pump
pA_2	The negative logarithmic value for antagonist coefficient derived from Schild's plot
PD	pharmacodynamic
pEC_{50}	- log(EC_{50})
pERK	Phospho-ERK
PK	pharmacokinetic
PKA	protein kinase A
PKCγ	protein kinase C gamma
PLH	planter heat test
PMA	phorbol 12-myristate 13-acetate
Ref	reference
REST	repressor element-1 silencing transcription factor
RIN-5F	pancreatic β-cells

s	seconds
s.c.	subcutaneous
SD	standard deviation
SD	Sprague Dawley (rat)
SEM	standard error mean
sl	serial number
SNc	substantia nigra pars compacta
SNL	spinal nerve ligated
STZ	streptozotocin
SW	Swiss Webster
t.i.d.	three times daily
TB	trypan blue (assay)
TF	tail flick test
TFL	tail flick latency
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TIPP	H-Tyr-Tic-Phe-Phe-OH
TRPV1	transient receptor potential vanilloid 1
TTX-R Na^+	tetrodotoxin-resistant sodium
T1DM	diabetes mellitus type 1
T2DM	diabetes mellitus type 2
UFP-505	H-Dmt-Tic-Gly-NH-Bzl
UTA	University of Tasmania
VE	vehicle
VFF	von Frey Filament

VTA	ventral-trigeminal area
WHO	World Health Organization
wks	weeks
WT	wild-type
WWTW	warm water tail withdrawal

Major terminologies used in this study

Motor behaviour	Horizontal locomotor activity, vertical locomotor activity (rearing) and turning behaviour
Motor suppression	Suppression of motor behavioural effects
Mixed activity	Selectivity for multiple opioid receptors
Tolerance	A reduction of an effect after repeated administration of an opioid

Abstract

Development of antinociceptive tolerance in the clinic after repeated administration of morphine limits its chronic use. Despite knowledge about the molecular mechanisms of morphine tolerance, we know little about the influence of dosage regimen (starting dose, follow-up dose, dosing and duration of treatment) for its development. I hypothesised that morphine dose, as well as dose increments, contribute to tolerance development. In addition, morphine-induced behavioural changes also might follow a similar pattern of antinociception and tolerance. Four groups of male Sprague Dawley rats received different daily doses of intermittent subcutaneous morphine for 14 days. After the development of antinociceptive tolerance, different increments of morphine doses were administered until tolerance redeveloped. Animals treated with lower starting-doses of morphine developed antinociceptive tolerance faster than those started on higher doses. Higher starting-doses and higher dose-increments after tolerance development resulted in more sustained antinociception and delayed the re-development of tolerance. These results were replicated in two anti-nociceptive assays and were therefore not assay-specific. The kinetics of morphine-induced motor suppression and desensitisation were similar to those of antinociception and antinociceptive-tolerance respectively. Overall, morphine dosing regimen in rats appears to significantly influence antinociceptive tolerance and total antinociception. My results also indicate that repetitive morphine dosing leads to desensitisation of motor suppression in all major motor behavioural parameters and manifests behavioural tolerance in conjunction with antinociceptive tolerance. Therefore, the results highlight that an optimised morphine dosing strategy can delay antinociceptive tolerance and reduce behavioural adverse effects.

Morphine and most other clinical opioids are MOP receptor agonists. The MOP receptor is responsible for both antinociception and generation of antinociceptive tolerance. Previous studies showed that an opioid ligand with mixed activity on multiple opioid receptor can reduce antinociceptive tolerance compared to morphine or other clinical opioids. Several novel opioids synthesised at the University of Tasmania were characterised for their *in vitro* specificity for major opioid receptors (MOP, DOP, KOP and NOP receptors) before one selected mixed activity opioid was tested for its antinociceptive effect, antinociceptive tolerance and motor behavioural effects *in vivo*. Collectively, UTA1003 acted as a MOP and KOP receptor agonist and a DOP receptor partial agonist. Therefore, I expected the ligand to reduce tolerance and motor suppression over a repeated treatment regimen. In rats, UTA1003 showed a mild antinociceptive effect with no noticeable motor suppression after subcutaneous administration. After repeated treatment over a period of eight days, UTA1003 displayed no tolerance and no motor behavioural adverse effect. In addition, the ligand maintained approximately 50% antinociception over the 8 days treatment without affecting morphine-induced motor suppression or hyper-excitation. Therefore, my study showed that co-administration of morphine and an opioid with mixed activity profile on multiple receptors can delay antinociceptive tolerance.

Worldwide, the elderly suffer from chronic pain while being the highest users of opioids. In current clinical practice, morphine is dosed in older patients based on patient-weight, with different calculations for adjustment. However, neither clinical experience nor the literature offers a clear evidence base for the relationship between antinociception, behavioural effects and morphine administration in older patients. In this study, I compared the nociceptive response of 8 and 24 week old rats after subcutaneous administration of morphine per body weight and analysed their motor behaviour. Residual morphine in all major tissues was determined. I observed prolonged morphine-induced antinociception in older rats compared

to younger rats. Moreover, morphine significantly stimulated locomotor and rearing behaviour 180 min after injection, which was significantly higher in the 8-week compared to 24-week old rats. Tissue analysis from animals extracted during the stimulatory phase revealed a significantly higher concentration of residual morphine in the brain of older versus younger animals when standardised on tissue weight. Collectively, morphine exhibited higher antinociception and increased behavioural inhibition in older compared to younger animals, likely due to the significantly higher accumulation of morphine in the brain of older animals. Therefore, my findings would suggest that a lower dose of morphine is sufficient to provide sufficient pain-relief in older patients.

Diabetes patients increase worldwide and elderly people are highly susceptible for diabetes mellitus. Apart from pain-relief and behavioural effects, opioids also affect insulin secretion in pancreatic β-cells. Therefore, a pancreatic β-cell line (RIN-5F) was exposed to different selective agonists and antagonists of the major opioid receptors. My findings suggest that MOP and DOP receptors are mainly responsible for pancreatic insulin release. Therefore, my findings along with previous studies contribute to a better treatment strategy for the management of insulin homeostasis and diabetic neuropathy.

Overall, proper dosing of an opioid can show better analgesia, less tolerance and less behavioural adverse effects. My study identified “age of patient” as an important parameter for opioid dosing. The novel opioid UTA1003 could serve as an adjuvant with morphine to reduce morphine tolerance over a long-term treatment. Follow-up studies with structural modifications are required to increase its potency as a pain-killer as well as a pharmacokinetic study would help to understand its mechanism of action.

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CHAPTER ONE

Introduction

1. Introduction

1.1. Opioid system

According to International Association for the Study of Pain (IASP), pain is a distressing sensory experience derived from a tissue damage (1). Pain can be categorised as nociceptive, neuropathic or a combination of both (mixed). Nociceptive pain is generated as a warning signal to inform about the possible damage of a non-neural tissue (1,2). In contrast, neuropathic pain usually results from damage to a neural tissue by a disease, toxin or an infection (1,3). A combination of nociceptive and neuropathic pain is commonly observed in situations like cancer and other indications that are characterised by long-term pain.

The opioid system is a physiological control system that modulates pain, emotions, immune defence and various other physiological responses. The opioid system involvesthe communication and coordination of a significant number of endogenous opioid peptides and several types of opioid receptors in the central and peripheral nervous system. This system also significantly modulates numerous sensory, emotional, motivational, cognitive functions as well as addictive behaviours (4,5,6). It is also involved in other physiological functions, including responses to stress, respiration, gastrointestinal transit, as well as endocrine and immune functions (7). These responses are orchestrated by opioids that bind to specific opioid receptors to induce analgesic and behavioural effects *in vivo*. Therefore, to understand the pharmacological effects of specific opioids, it is essential to first clarify the specific roles of each opioid receptor.

1.1.1. Opioid receptors and pain-relief

Analgesic responses are regulated by different neuronal signals, which are controlled by different types of opioid receptors. The presence of opioid receptors was first proposed in 1954 (8). However, the first evidence for different types of opioid receptors was only described in 1976 (9). According to the International Union of Basic and Clinical Pharmacology (IUPHAR) and the British Pharmacological Society (BPS) joint *IUPHAR/BPS Guide to Pharmacology*, opioid receptors are classified into μ (Mu: MOP), δ (delta: DOP) and κ (kappa: KOP) receptors as well as the non-classical opioid (NOP) receptors (10) (Table 1).

Opioid receptors belong to the family of seven-transmembrane helical G protein-coupled receptors (GPCRs). These receptors display an extracellular N-terminus and intracellular C-terminus and are coupled with heterotrimeric G_i/G_o proteins (11,12,13). Opioid ligands bind to opioid receptors by ligand-receptor interactions in the binding pockets of the receptor, which are situated in the transmembrane helices. The binding pocket of opioid receptors can be divided into two distinct regions: the lower part (intracellular side) is highly conserved for opioids (non-specific 'message'-region) and the higher part of the pocket (extracellular side) contains divergent residues that confer selectivity ('address' region) of opioid receptor types and binding depends on the type of the opioid ligand as well (14,15). In 2012, the molecular structures of all four opioid receptors were described by several reports (14,16,17,18) and overall displayed around 60% similarity.

Although all of these opioid receptors (OR) modulate antinociception, the MOP receptor is thought to be dominant for its antinociceptive effects *in vivo* (19,20,21,22). The major limitation of targeting the MOP receptor for analgesia is that it is also responsible for the induction of tolerance (23) and other side-effects like respiratory depression (24) or constipation (25). MOP receptor-related adverse events are of great clinical concern and justify

the characterisation of other opioid receptors as suitable drug targets to induce analgesia. Unfortunately, the other three receptors do not show the same efficacy to transmit drug-induced analgesia compared to the MOP-receptor. DOP-receptor agonists are generally less effective to treat acute thermal pain compared to inflammatory (26,27,28), neuropathic (29,30) and cancer -associated bone pain (31). SNC80 and Deltorphin II (both DOP receptor agonists) showed significant anti-hyperalgesic effects, but these compounds were less potent or less efficacious to induce thermal antinociceptive effects (27). In addition, the use of DOP-receptor agonists is limited since DOP receptor-induced analgesia appears to require the presence of a pro-inflammatory state (32,33). While DOP-receptor agonists only produce moderate analgesia in non-human primates (34,35,36), despite being effective in rodent models of chronic pain (37), they are associated with convulsions in mice (38) and non-human primates (34,35,36). KOP-receptor agonists are reported to reduce inflammatory (39) and neuropathic pain (40) to some extent but are limited to non-systemic use to avoid CNS-associated adverse events (41,42,43). While selective KOP or DOP receptor agonists lack some of the MOP receptor agonist- (e.g. morphine) associated side-effects such as constipation, respiratory depression and addiction, they display a side-effect profile of their own that includes diuresis, sedation and dysphoria (45). In a recent study, two KOP receptor agonist ligands, HS665 and HS666 showed dose-dependent thermal antinociception similar to U50,488 after i.c.v. administration (44). The authors showed that the effects were mediated by the KOP receptor and HS665 did not show any dysphoric effects, as it showed low efficacy in the β-arrestin2 signalling pathway (44). Several NOP receptor agonists are reported to show antinociceptive effects in rodent (46,47) and primate models (48,49,50) and are associated with a reduced risk for abuse (51). However, systemic administration of these NOP agonists did not produce spinal analgesia in rodents (52,53), while showing efficacy using intrathecal administration in primates and rodent models of neuropathic pain (47,48,51,54). Overall, MOP-receptor agonists, despite their

adverse-effects, therefore appear superior to provide pain relief and are thus widely used in the clinic (22,55).

Table 1. Classification of opioid receptors. Keys: β-funaltrexamine (β-FNA), [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), norbinaltorphimine (norBNI), [D-Pen2,D-Pen5]-enkephalin (DPDPE), nociceptin/orphanin-FQ (N/OFQ), H-Tyr-Tic-Phe-Phe-OH (TIPP).

Receptor Nomenclature[1]	**Gene**	**Most common location in the CNS[2]**	**Most common roles and functions**	**Selective Agonist [3]**	**Selective Antagonist[4]**
μ, mu, MOP	OPRM1	thalamus, amygdala, dorsal horn, cerebral cortex, striatum, hippocampus, locus coeruleus	analgesia, intestinal transit, feeding, mood, hormone secretion, thermoregulation, cardiovascular function	DAMGO, sufentanil, PL017,	CTAP, CTOP, β-FNA,
δ, delta, DOP	OPRD1	olfactory bulb, thalamus, cortex, caudate putamen, nucleus accumbens, amygdala, dorsal horn	analgesia, mood, gastrointestinal motility, behaviour, cardiovascular regulation	DPDPE, [D-Ala^2]deltorphin I , [D-Ala^2]deltorphin II, SNC80	Naltrindole, TIPPΨ, Naltriben
κ, kappa, KOP	OPRK1	olfactory bulb, nucleus accumbens, cerebral cortex, claustrum, amygdala, caudate nucleus, hypothalamus, subthalamic nucleus, thalamus, corpus callosum.	analgesia in inflammation, diuresis, feeding, neuroprotection, neuroendocrine functions	Enadoline, U50488, U69593, salvinorin A	norBNI, GNTI
N/OFQ, NOP	OPRL1	hippocampus, hypothalamus, amygdala, substantia nigra, dorsal horn, lateral septum	spinal analgesia, anxiety, mood, memory, feeding, locomotor activity	UFP-102, Ro64-6198, N/OFQ-(1-13)-NH_2, UFP-112	UFP-101, SB 612111, J-113397, JTC-801

[1]Recommended and alternative nomenclature for opioid receptors as defined by IUPHAR/BPS guide to pharmacology (http://www.guidetopharmacology.org).
[2]For full information on receptor location in the CNS, see the IUPHAR/BPS database on http://www.guidetopharmacology.org
[3,4] As published by Alexander and colleagues (56) *and* from *IUPHAR/BPS receptor database (http://www.guidetopharmacology.org).*

To investigate the role of specific opioid receptors or ligands in pain modulation, antinociceptive tolerance and adverse behavioural effects, knock-out animals are frequently used. It is not surprising that opioid ligands show different effects in particular opioid receptor knock-out mice. For example, in MOP receptor knock-out mice, MOP receptor agonist-induced antinociception and their associated side-effects (e.g. hyperlocomotion, respiratory depression, inhibition of GI transit, reward and withdrawal effect) were effectively abolished (19,57,58). At the same time, morphine efficiently induced analgesia in DOP (59) and KOP (60) receptor knock-out mice, albeit with reduced side-effects (tolerance and withdrawal response). Similarly, KOP receptor agonists are also reported to induce analgesia in MOP (61) and DOP (59) receptor knock-out mice, while predictably in KOP receptor knock-out animals this effect was not observed (60). Similarly, DOP receptor agonists show only reduced levels of analgesia in DOP receptor knock-out mice (59), although a mixed effect (decreased/maintained) on analgesia was observed in MOP receptor knock-out mice (61,62).

1.1.2. Opioid ligands

A ligand is a compound that can form complex with a protein to initiate a biological effect. Opioid ligands (OL) are from both endogenous and exogenous origins. Once an agonistic ligand binds a receptor, it activates a G-protein (a trimeric protein has three different subunits (α, β and γ)) and dissociates the α unit to activate different downstream transduction pathways such as adenylyl cyclase (63). This activation modulates voltage-gated calcium, sodium and potassium channels (64,65) and cellular levels of cyclic adenosine monophosphate (cAMP) or protein kinase A (PKA) (66). Moreover, these receptors can signal through different pathways, such as β-arrestin (67,68), which can vary according to the chemical properties of the ligand (69). Opioid peptides activate receptors on sensory nerve fibres and produce acute analgesia by reducing the excitability of sensory neurons. MOP receptor activation by both endogenous

and exogenous opioids in post-synaptic neurons dissociates the G_{α} subunit from the $G_{\beta\gamma}$ subunits of the G protein which increases potassium conductance in neurons. The resulting efflux of potassium ions (K^{+}) hyperpolarise the neuronal cells and reduces their excitability (70,71,72). However, chronic opioid treatment can induce hyperalgesia. This adverse effect is associated with G_{α}-induced hyper-excitability of tetrodotoxin-resistant sodium channels (TTX-R Na^{+}) and transient receptor potential vanilloid 1 (TRPV1) channels of the dorsal root ganglia neurons (73,74). Therefore, activation of opioid receptors by endogenous and pharmacological molecules can differentially alter neuronal excitability by modifying different ion channels after acute and chronic treatment.

The signalling processes initiated by the activation of opioid receptors also include gene transcription events that control cell differentiation, proliferation and survival (70,75). When an opioid ligand binds with an opioid receptor, a G_{α} subunit is dissociated and activates adenylyl cyclase to synthesise cyclic adenosine monophosphate (cAMP). cAMP binds with regulatory proteins of protein kinase A (PKA) and activates PKA. The activated PKA translocate to the nucleus where it induces the phosphorylation of cAMP response element binding protein (CREB). The phosphorylated CREB facilitates desired gene expression levels as the promoters contains cAMP response elements (CRE) and control different cellular functions (76). Increased activity of CREB was observed in cancer cells and blocking CREB can block cell proliferation, differentiation and survival (77,78). However, another study shows CREB has dual role in cell proliferation (simulate or inhibit) and its role depends on the way of its activation (79). For example, cAMP activated CREB stimulates mitosis (cell proliferation) but growth factor activated CREB inhibits mitosis (79). Prolonged administration of morphine reduces both MOP and NOP receptor gene expressions, while prolonged exposure of fentanyl induces upregulation of MOP receptor gene expression without affecting NOP

receptor gene expressions (80). A KOP receptor agonist dynorphin B also showed to increase opioid peptide gene transcription (81). Therefore, opioid receptor activation by a ligand can lead to gene transcription events in neuronal cells.

Several chemically distinct compounds (ligands) can activate opioid receptors and these ligand-receptor interactions result in cellular adaptation and altered physiological outcome. This phenomenon is termed as biased agonism (82,83,84,85). Biased agonism of MOP receptor is studied to avoid antinociceptive tolerance and other adverse effects with particular focus on the development of ligands with no β-arrestin-2 (β-arr2) recruitment (86,87,88). Increased antinociceptive response and reduced adverse effects were observed in ligands with decreased β-arr2 recruitment or in β-arr2 knockout mice (88,89,90,91,92,93). Biased agonists for the KOP receptor with reduced β-arr2 recruitment also showed antinociception with less adverse effects compared to selective KOP receptor agonists (44). The development of biased ligands are at a preclinical stage and it is expected that in the clinic these drugs will be safer than conventional opioids (83).

1.1.2.1. Endogenous opioid ligands

Evidence for endogenous ligands of opioid receptors was obtained in the 1970s and the structures of [Leu]enkephalin and [Met]enkephalin were reported in 1975 (94). Endogenous opioid peptides are found in the central and peripheral nervous system and the gastrointestinal tract (95). These peptides are derived from the four different precursors pro-enkephalin, pro-dynorphin, pro-opiomelanocortin, and prepro-nociceptin (11,96,97,98). Pro-enkephalin contains two hundred sixty seven amino acid polypeptides (99) and mainly produce the pentapeptides [met]- and [leu]-enkephalins (100,101) with a selectivity for MOP and DOP receptors. Dynorphins are mainly big dynorphin, dynorphin A, dynorphin B and α-Neo-endorphin and interact mainly with the KOP receptor (102,103). Endorphins are derived from

pro-opiomelanocortin (104) and are expressed as α, β and γ- endorphins (105). While endorphins activate the MOP receptor, the prepro-nociceptin derived neuropeptide nociceptin/orphanin FQ binds to the NOP receptor (5). Endogenous opioids affect a multitude of physiological functions such as pain modulation and analgesia, stress and emotional responses, tolerance and dependence, learning and memory, addiction, sexual activity and control of hormone levels, neurological disorders, eating and drinking behaviour, gastrointestinal, renal and hepatic functions, cardiovascular responses, respiration, thermoregulation and immunological responses (106,107).

1.1.2.2. Exogenous opioid ligands

Over a period of approximately 8000 years, the poppy plant (*Papaver somniferum*) and the opioids derived from it have been used for pain relief. In a Sumerian ideogram, the poppy plant was known as a "plant of joy"(107). Crude opium admixtures were widely used in different British and German medicines from the 16th century and effects like pain tolerance and physical dependence to opioids were noted at this time. In 1805, Friedrich Sertürner isolated morphium (morphine), named after the Greek god Morpheus (god of sleep and dreams). Within two decades after the initial isolation of morphine, commercial production of morphine started and became available on the European market. Subsequently, after the invention of hypodermic syringes in the middle of the 19th century, morphine was injected locally into painful areas (107).

Currently, different alkaloid extracts from the poppy plant (*Papaver somniferum*), such as opium, morphine and codeine are still used for pain relief, mood disorders and palliative care. In addition, several semi-synthetic and synthetic opioids, such as buprenorphine, dextropropoxyphene, hydromorphone, oxycodone, pethidine, fentanyl, methadone, tapentadol and tramadol are widely used in patients that suffer from surgical or chronic pain (108).

1.1.3. Clinical use of opioids

Opioid drugs are typically receptor-specific and display different advantages as well as adverse effects in clinical use. Opioids are very effective against moderate to severe intensive acute, surgical and chronic pain. Most of the clinically used opioids are MOP receptor agonists and are associated with a significant number of side-effects such as tolerance, dependence, desensitisation or withdrawal symptoms (109). The use of clinical opioids in a particular patient depends on the severity of pain and the adverse effects profile of that particular drug. Currently, morphine sulphate, morphine hydrochloride, oxymorphone hydrochloride, hydromorphone hydrochloride, hydrocodone bitartrate, fentanyl, tapentadol hydrochloride, methadone hydrochloride, buprenorphine, naltrexone hydrochloride, naloxone hydrochloride and some other opioids are available for human use, either alone or in combination as an immediate release or controlled release formulations (108,110).

1.1.4. Opioid-induced adverse-effects

1.1.4.1. Analgesic tolerance

The development of analgesic tolerance of opioids after repetitive administration is one of the major limitations for their chronic use in the clinic. Morphine is one of the most effective and widely prescribed drugs against chronic pain (22). However, long-term morphine treatment is discouraged in the clinic due to the risk of adverse side-effects including analgesic tolerance (111,112). Tolerance manifests as decreased drug efficacy following repeated administration (113). Therefore to maintain efficacy dose-increments are required, which in turn contribute to generate additional adverse effects. In the clinic, patients frequently receive inadequate doses of opioids, due to an insufficient understanding regarding opioid formulation, dose titration and analgesic tolerance (114). In addition, increased morphine dosing is frequently required due to increased disease progression, rather than analgesic tolerance (115).

The clinical management of analgesic tolerance involves opioid rotation and the combination of opioids with adjuvants (116,117,118,119). Adjuvants, like gabapentin, pregabalin, dexamethasone, naproxen, ibuprofen, carbamazepine, aspirin, venlafaxine and acetaminophen are combined with opioid analgesics in patients that require long-term analgesic treatment (120,121). Similarly, in preclinical studies, a combination of opioids and non-opioid adjuvants or combinations of opioid agonist and antagonist are used to prevent antinociceptive tolerance (122,123,124,125). In response to an opioid ligand binding to an opioid receptor, inhibition of adenylyl cyclase (AC) reduces the levels of the second messenger cAMP (86,130,131). In addition, activation of opioid receptors leads to their phosphorylation by GPCR kinases, which promotes the interaction with β-arrestins (βarr) (130,131). Both phosphorylation and interaction with βarr are required for subsequent receptor internalisation (130,131). This internalised receptor can be proteolytically degraded. However, some receptors can also be

recycled in endosomes to be returned to the cell membrane (131,132). This process in called receptor trafficking. In addition, de novo receptor synthesis ensures that new opioid receptors are produced and transported to the cell membrane via the trans-Golgi network (131). Prolonged treatment with opioids either increase the numbers of inactive (phosphorylated) receptors in the membrane or increases the number of de novo produced receptors (131,132).

Specifically, chronic exposure to morphine leads to the selective recruitment of β-arrestin-2 (βarr2) but not β-arrestin-1 (βarr1) (68). In contrast to the interaction with βarr1, which leads to receptor recycling, βarr2 does not lead to opioid receptor recycling but increases the number of inactive receptors in the cell membrane. This process is associated with insufficient analgesia (68). Although the molecular mechanisms that leads to opioid tolerance are not entirely clear, both desensitisation and trafficking are suspected to be the key factors that lead to insufficient analgesia (68,86,131,133). Although it is important to delineate the exact molecular mechanisms leading to opioid tolerance (68,126,127,128,129), it is also important to understand how chronic morphine dosing itself can influence tolerance (chapter 2).

Pain-relief or analgesia is measured in preclinical studies as nociceptive and neuropathic pain. The antinociceptive effects of morphine and other opioids in preclinical studies are commonly measured as central (brain and spinal cord) or peripheral antinociception (134,135). The commonly used tail-flick test potentially measures the spinal-mediated nociception while the hot-plate assay largely measures a supra-spinal-mediated nociception (135,136). Generally, one antinociception test is performed in preclinical studies with repeated morphine treatment (Table 2). As a result, the progression of antinociceptive tolerance measured by a single assay may be different when using another assay (137). Therefore, more than one antinociception assay is required to represent the true extent of antinociceptive tolerance.

Table 2. Preclinical Analgesic tolerance studies of morphine. Keys: M.HCl: morphine hydrochloride, MS: morphine sulphate, M: morphine base, TF: tail flick test, WWTW: warm water tail withdrawal test, HP: hot plate test, PLH: planter heat (by radiation) test, VFF (von Frey test, mechanical allodynia). i.t.: intrathecal, i.c.v.: intracerebroventricular, i.v.: intravenous, s.c.: subcutaneous, os.pump: osmotic pump, wks: weeks, D: day.

Sl	Assay (^oC)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
1	WWTW (52^oC)	MS (s.c.)	Once daily for 14 days	7.52	7	Latency	Wister rats (220-260 g)	12	3-5	(138)
2	HP (58^oC)	MS (s.c.)	M. pellets for 7 days (induction); tested with MS (s.c.) every day	75 mg (for induction); 11.28 (test)	3 (60 min)	Latency	Holtzman rats (150-200 g)	30	-	(139)
3	HP (55 ^oC)	MS (s.c.)	Twice daily for 5 days (tested days: 1, 6)	7.52	6 (15,30 and 60 min)	MPE%	Swiss mice (25-30 g)	15	-	(140)
4	HP (55 ^oC)	MS (s.c.)	Twice daily for 5 days (tested days: 1, 6)	7.52	6 (30 min)	MPE%	Swiss mice (25-30 g)	45	-	(141)
5	HP (54 ^oC)	MS (s.c.)	Once daily for 8 days (tested days: 1,4,8)	7.52	8 (30 min)	MPE%	SD rats (200-250 g)	45	-	(142)
6	HP (55 ^oC)	MS (s.c.)	Once daily for 8 days (tested daily)	7.52	8 (30 min)	MPE%	C57Bl/6 mice (8-10 wks)	30	-	(143)
7	HP (54 ^oC)	MS (s.c.)	Once daily for 7 days (tested days: 1, 4, 7)	7.52	7 (60 min)	MPE%	SD rats (275-300 g)	45	-	(144)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
8	TF	MS (s.c.)	Twice daily for 7 days	7.52	5 (30 min)	MPE%	C57BL/6 Mice	12	2-4.5	(145)
9	WWTW (52 °C)	MS (s.c.)	Twice daily for 5 days (tested days: 1, 6)	7.52	6 (30 min)	MPE%	ICR mice (21-25)	12	-	(146)
10	TF	MS (s.c. os.pump)	Continuous delivery for 4 weeks (induction); tested once per week	7.52 or 15.04 /day (induction); 7.52 (test)	7	ED_{50}	SD rats (250-275 g)	6	2-2.5	(147)
11	TF	MS (s.c.)	Once daily for 7 days (or, morphine base pellets for 7 days)	13.91 - 139.12 (or, 25 mg, 50 mg and 75 mg)	7 (or, 2 for pellets)	ED_{50}	SW mice (26-40 g)	10	1-3	(148)
12	TF	MS (s.c.)	Twice daily for 6 weeks (tested once a week)	3.76, 7.52, 15.04, or 30.08 (4 groups)	7 (all groups)	ED_{50}	CD1 mice	10	-	(149)
13	TF, HP (53 °C)	MS (s.c.)	Twice daily for 5 days	11.28	5	MPE%, ED_{50}	SW mice (21-30 g)	10 (TF); 30 (HP)	2-4 (TF); 8-10 (HP)	(150)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
14	WWTW (55 °C)	MS (s.c.)	Twice daily for 3 days (induction), (tested days 1 and 4)	ED_{90} dose (unknown) of MS (induction), ED_{50} dose (test)	4	ED_{50}	ICR mice (25-35 g)	10	<5	(151)
15	TF	MS (s.c.)	Once daily for 9 days (tested days: 1,2,4 and 9)	7.52	9	MPE%	ICR mice (25-30 g)	15	4.32± 0.12	(152)
16	TF	MS (s.c.)	Three times daily for 20 days; tested days: 1 and 21	Started with 7.52 and increased continuously 3.76-7.52 (in every 2 days) and last 2 days (112.8)	21	ED_{50}	SW mice (20-23 g) (both sexes)	-	1.1± 0.2	(153)
17	TF	MS (i.p.)	Three times daily for 3 days (induction); tested day: 4	37.6, 37.6 or 56.4 (induction); 5.26 (test)	4	Latency	Mice (25-30 g)	10	-	(154)
18	WWTW (55 °C)	MS (i.p.)	Twice or thrice daily for 3 days; tested day: 1, 4	4.51 (induction); 1.73 (test)	4	ED_{50}	ICR mice (20-30 g)	15	< 5	(155)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
19	WWTW (52 °C)	MS (i.t.)	Continuous delivery for 5 days by osmotic pump (induction), tested days: 1,5	11.28 µg/h (induction); 11.28 µg (test)	5	MPE%, latency	Wister rats (350-400g)	10	2.0± 0.3	(156)
20	HP (52.5 °C)	MS (vlPAG)	Twice daily on ventrolateral periaqueductal gray (vlPAG) for 3 days. Tested days:1,4	3.76 µg	4 (15 min)	ED_{50}	SD rats (200-340 g)	50	-	(157)
21	TF	MS (i.t.)	Twice daily for 6 days; tested days: 1,3,5 and 7	7.52 µg	5 (30 min)	MPE%, ED_{50}	SD rats (300-350 g)	10	4-5	(158)
22	TF	MS (i.t.)	Once daily for 8 days	7.52 µg	8	MPE%	SD rats (350-400 g)	10	3.5-4.5	(159)
23	WWTW (55 °C)	MS (i.c.v.)	Twice daily for 3 days, tested days: 1,4	ED_{90} (not mentioned) (induction); 0.43 µg (test)	4	ED_{50}, MPE%	ICR mice	10		(160)
24	PLH	MS (i.t.)	Twice daily for 4 days, tested days: 1, 5	15.04 µg	5	MPE%, latency	SD rats (250-300 g)	20	-	(161)
25	PLH (30 °C)	MS (i.t.)	Once daily for 7 days	11.28 µg	7	MPE%	SD rats (200-250 g)	20	-	(162)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
26	VFF	MS (i.t.)	Once daily for 7 days, tested every day	15 µg	5 (30 min)	PWT	SD rats (200-250 g)	-	-	(163)
27	TF	M.HCl (s.c.)	Twice daily for 5 days	53.20	6	MPE%	Mice (6-8 wks)	6	2.7-3.3	(164)
28	TF	M.HCl (s.c.)	Once daily for 5 days	8.87	3	AUC	ddY mice (18-20g)	10	–	(165)
29	TF	M.HCl (s.c.)	Twice daily for 5 days; tested days: 1, 3 and 5	8.87	3	MPE%	CFLP white mice (30±5 g)	20		(166)
30	TF, HP (52° C)	M.HCl (s.c.)	Twice daily for 4 days	8.87 (D1), 13.3 (D2), 17.74 (D3), 26.61 (D4) (induction); 4.44 (test)	5 (60 min)	MPE%	SD rats (300 g)	10 (TF); 60 (HP)	2.1±0.5 (TF); 9.7±3.8 (HP)	(167)
31	TF	M.HCl (s.c. implant)	Continuous delivery for 5 days	5.32	5	Latency, MPE%	SD rats (300-350 g)	10	6 -	(168)
32	TF	M.HCl (s.c. implant)	Once daily for 5 days	8.87	5	AUC	ddY mice (4-5 wks)	10	2-3	(169)
33	TF	M.HCl (s.c.)	Twice daily for 7 days	8.87	5	MPE%	Wister rats (200-250 g)	10	2-4	(170)
34	TF	M.HCl (s.c.)	Twice daily for 5 days (test days: 1, 3, 5)	7.09	3	MPE%	ddY mice (25-33 g)	10 (TF),	-	(171)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
								30 (HP)		
35	HP (52°C)	M.HCl (s.c.)	Once daily for 10 days	2.22	10	MPE%	Swiss mice (25-30 g)	240	-	(172)
36	TF	M.HCl (i.p., i.t.)	Once daily (i.p.) for 3 days; Osmotic pump (i.t.)	8.87 (i.p.); 13.30 µg/h (i.t.)	3 (i.p.); 5 (i.t.)	AUC, MPE%	SD rats (250-275 g)	15	-	(173)
37	WWTW (52°C)	M.HCl (i.p.)	Twice daily for 9 days	13.3 (day: 1, 9); 13.3 or 26.6 (day: 2-8)	No tolerance; 9th day (45 min)	Latency	C57 mice (wild type)	10	-	(174)
38	WWTW (50 °C)	M.HCl (i.t.)	Once daily for 7 days	13.3 µg	7	MPE%	SD rats (250-280 g)	15	-	(175)
39	TF	M.HCl (i.t.)	Once daily for 7 days	17.73 µg	7	MPE%	SD rats (250-320 g)	10	2-3	(176)
40	HP (55°C)	M.HCl (i.t.)	Twice daily for 7 days (test days 1,8)	8.87 µg	8	Latency	SD rats (150-200 g)	30	3.8	(177)
41	TF	M.HCl (i.t.; i.c.v.)	Once daily for 8 days (tested daily)	3.55 nmol (i.t.); 1.77 nmol (i.c.v.)	4 (30 min i.t.; 15 min i.c.v.)	MPE%	Kunming mice (18-22 g)	10	3-5	(178)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
42	TF	M pellets (s.c.)	Continuous delivery until 6th day; tested days: 0.5, 1, 2, 3 and 6.	75 mg	1 (24 h or after)	ED_{50}	SW mice (20-23 g) (both sexes)	-	1.1± 0.2	(153)
43	TF	M pellets (s.c.)	Continuous delivery for 7 days; tested days 1 and 7 with MS s.c.	25 or 75 mg (induction); 0.5, 1.0, 2.0, 3.0 (test)	7	ED_{50}	SW mice (22-44 g)	10	2-4	(179)
44	WWTW (48 °C)	M (s.c.)	Once daily for 7 days	5	7	Latency	C57BL6 mice (25-30 g)	25	-	(180)
45	PLH	M** (s.c.)	Twice daily for 10 days; tested days: 1,6 and 11	10	7 (30 min)	MPE%	SD rats (200-250 g)	20	-	(181)
46	TF	M** (s.c.)	Twice daily for 6 days, tested on days: 1, 7.	10	7 (30 min)	MPE%	SD rats (200-250 g)	15	6-8	(182)
47	TF, PLH,VFF	M** (i.t.)	Twice daily for 6 days	10 µg (induction); 0.25 µg (test)	7 (30 min)	MPE%	SD rats (200-250 g)	-	-	(183)
48	WWTW (55 °C)	M** (i.p.)	Once daily for 5 days	4	5	MPE%	C57Bl6 mice (25-28 g)	10	-	(184)
49	VFF	M** (s.c.)	Twice daily for 9 days	10	5 (30 min)	PWT	Kunming mice (26-30 g)	-	-	(185)

Sl	Assay (°C)	Morphine used (route)	Treatment procedure	Adjusted morphine dose* (mg/kg or as stated)	Tolerance from day (time)	Tolerance: as changes in	Animal species (male, or as stated)	Cut-off Lat. [s]	Basal Lat. [s]	Ref.
50	WWTW (52 °C), VFF	M** (i.p.)	Twice daily for 4 days	10 or 20	4	MPE %	SD SNL rats (250-300 g)	10	3.95± 0.04	(186)
51	VFF	M** (s.c.)	Twice daily for 9 days; tested days: 0 and 9	10 (induction), 5 (test)	9	PWT	SD rats (220±10 g)	-	-	(187)
52	HP (52.5°C)	M** (vIPAG)	Twice daily for 2 days	5 µg	2 (15 min)		SD rats (250-350 g)	50	<20	(188)
53	WWTW(52.5°C)	M** (i.t.)	Twice daily for 7 days and tested everyday	15 µg	4 (30 min)	MPE%	SD rats (220-250 g)	10	-	(189)

*Notes: *Morphine dose was adjusted as morphine base. Conversion factor based on their molecular formula: Morphine in M. (base) (100%), in M.HCl (88.67%) and in MS. (75.20%). **There is no clear information on types of morphine (base, sulphate or hydrochloride). Only morphine is mentioned in these articles.*

1.1.4.2. Behavioural effects

In addition to antinociceptive tolerance as an adverse effect of long-term opioid treatment, the discussion also needs to include behavioural side-effects which are observed in the clinic (190). Morphine-induced biphasic behavioural effects are well known from preclinical studies and include initial motor suppression and subsequent hyper-excitation (191,192,193,194,195,196,197). An open-field arena is widely used to assess motor behaviour and typically includes horizontal movement, rearing (vertical movement) and turning behaviour. Morphine-induced horizontal locomotion, turning and circling behaviours are related to the dopaminergic system (194,198,199,200). Morphine treatment induces the dopamine receptor-1 (D1) dependent beta-arrestin-2 (βarr2) / phospho-ERK (βarr2/pERK) signalling complex, which regulates morphine-induced locomotion (201). Acute morphine administration induces phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), which activates the D1 receptor in neurons of nucleus accumbens and dorsal striatum and alters locomotor activity (201,202,203,204). In contrast, the effects of chronic morphine treatment on the cAMP/PKA/DARPP-32 signalling are not fully understood at present. However, rearing behaviour can indicate anxiety, exploration or sedation, which are related to gama-aminobutyric (GABA) inhibitory neurotransmission (205,206,207). The activation of the cAMP-responsive element binding protein (CREB) transcription factor regulates anxiety-related behaviours, as CREB-deficient mice showed an increased anxiogenic response (208). However, the behavioural changes in response to chronic morphine treatment are independent of the MOP receptor, cyclin-dependent kinase 5 (cdk5) or adenylate cyclase activities in areas of brain relevant (209). The likely multiple mechanisms that link chronic morphine-treatment with its behavioural effects are not completely understood, but are probably controlled by a combination of dopaminergic, GABA-ergic, opioidergic and additional unknown neuronal signals (86,201,202,203,205,206,207).The combination of

multiple independent behavioural measurements is generally regarded as the most reliable approach to assess the total motor effects induced by opioids (192,193,210).

1.1.4.3. Constipation

Constipation is a very common side-effect of opioids and it is caused by activation of MOP receptor in the enteric nervous system (211,212). Opioids bind with MOP receptors in the enteric neurons and delay gastrointestinal (GI) transit time, which also stimulates non-propulsive GI motility, pylorus and ileocecal sphincters. Morphine treatment increases expression of aquaporin-3 (AQP3) water channels in the colon by increased secretion of 5-HT (serotonin), which increases water absorption from luminal part to the vascular part of the colon (213). As a result, constipation develops by increased fluid absorption from the large intestine along with less electrolyte secretion by intestinal lumen (212). In contrast, chronic morphine treatment does not produce tolerance to reduced gastrointestinal (GI) motility in the lower GI tract, while it induces analgesic tolerance and leaves GI motility unaffected in the upper GI tract (214). As a result, patients continuously suffer from constipation over a long-term opioid treatment. Constipation affects about 40% of patients with chronic oral opioid treatment and therefore different laxatives and non-medication approaches (e.g. fibrous diet, hydration) are used to provide comfort of the patients (215,216,217,218). In addition, opioids combined with a low dose of opioid antagonists like naloxone or methylnaltrexone are effective to reduce constipation without affecting pain-relief and inducing less withdrawal symptoms (218,219,220,221).

1.1.4.4. Nausea and vomiting

Nearly 20% of patients with long-term opioid treatment experience nausea and vomiting (222). The actual mechanism for opioid-induced nausea and vomiting is not clear, but the activation of opioid receptors (MOP or DOP) present in the chemoreceptor trigger zone, vestibular

apparatus (MOP) and GI tract (MOP, DOP or KOP) are probably involved in the induction of nausea and vomiting (130). At present, it is thought that these adverse effects are a direct consequence of opioid-induced effects in the *area postrema* of the brainstem, an area rich in dopamine, opioid and serotonin receptors (223,224). In the clinic, 5-HT_3 and NK_1 receptor antagonists are used to prevent opioid-induced emesis, which could indicate that several non-opioid receptors (e.g. dopamine (D_2), 5-HT_3 and histamine (H_1)) might interact with opioid receptors in those brain areas that control nausea and vomiting (130,225,226,227). Although patients treated with oral morphine experience chronic nausea and vomiting, opioid rotation or changing the route of administration (e.g. oral to subcutaneous) appear helpful to reduce these adverse effects (216,228,229).

1.1.4.5. Respiratory depression

Respiratory depression occurs less frequently compared to other adverse effects, but it typically can have fatal consequences in the clinic (217,230). Similar to the other side effects, opioid-induced respiratory depression is mediated by the MOP receptor (231,232,233). Neurons of the pre-Bötzinger complex, a sub-region of the ventrolateral medulla, are responsible to control autonomic neuronal functions including normal respiration (233). The neurons of pre-Bötzinger complex express a variety of receptors including neurokinin-1, serotonin (5-HT) and MOP receptors (233). MOP receptor activation inhibits AC and reduces synthesis of intracellular cAMP, which is thought to depress the respiratory neurons as reduced cAMP levels in cytoplasm reduces neuronal excitability by an unknown mechanism (130). On the other hand, serotonin receptors in this region stimulate respiration (234,235). 5-$HT_{1(a)}$ receptors are expressed widely in respiratory neurons and are activated by reduced cAMP levels activating the glycine receptor type α3 (GlyRα3) (236). The activated GlyRα3 receptor inhibits the inhibitory neurons that cause respiratory depression. This effect is independent of the MOP

receptor induced signal transduction pathway (236). Therefore, multiple non-opioid receptors together with the MOP receptor are involved in the control of respiration and opioid-induced respiratory depression. Although high dose opioid users are at risk of respiratory depression (237), selective opioid receptor antagonist can effectively reduce the incidence of respiratory depression without significant withdrawal symptoms (238).

1.1.4.6. Addiction and physical dependence

Physical dependence is the craving for a drug either for pleasure or to avoid the occurrence of withdrawal symptoms following a reduction of treatment dose or intake of an opioid receptor antagonist (217,239). Addiction indicates a loss of control of opioids use, despite adequate pain-relief (239). Opioid addiction is mediated by MOP receptors in the *ventral tegmental* area and the *nucleus accumbens* of the brain (240,241). Physical opioid dependence associated with upregulation of cAMP and noradrenergic signalling in the *locus coeruleus* neurons of dorsal pontine tegmentum of the brainstem (240,241,242). In contrast to acute exposure of morphine that reduces cAMP levels, chronic morphine administration increases AC levels type I and VII, PKA subunits, several phosphoproteins (e.g. CREB) and results in activation of the cAMP pathway (241). The actual molecular mechanism that initiates the beginning of physical dependence is unknown, but is associated with repeated opioid treatment over longer time intervals (240). Clinical guidelines for long-term opioid use propose a "start low and go slow" dosing regimen to prevent addiction, physical dependence, overdosing or abuse (111,243,244,245,246,247). Therefore, clinical guidelines propose the smallest effective dose (248), rather than aiming for adequate long-term pain relief (249).

1.1.4.7. Other adverse effects

Drowsiness, lethargy, hyperalgesia and pruritus are also common adverse effects of morphine (250,251,252). Drowsiness and lethargy are caused by a suppression of motor behaviour,

however the exact mechanisms for this effect are not known. Morphine is also responsible to generate an itching skin sensation by signalling through spinal heteromers of opioid- and itch-mediating GPCRs (253,254). Withdrawal after chronic morphine exposure also induces histamine-induced itching or scratching responses (255). Finally, morphine can induce increased pain sensation (hyperalgesia) via the MOP receptor (256). However, the molecular mechanism of morphine-induced hyperalgesia are not well-understood but might be related to the upregulation of protein kinase C gamma (PKCγ) and the NMDA receptor subtype NR1 in the spinal cord (257,258). It is also thought that different MOP receptor isoforms, functional interactions with other GPCRs or opioid metabolites such as morphine 3-glucuronide that interact with GABA or NMDA receptors could be responsible for this adverse effect (180,252,256).

1.2. Opioids with selectivity on multiple receptors

1.2.1. Receptor dimers and heteromers

Concomitant treatment of an opioid with an opioid receptor antagonist can reduce adverse effects, which indicates the interactions of multiple opioid receptors in the same cell (218,219,220,221,238). There is significant evidence that opioid receptors and other G-protein coupled receptors can form dimers or oligomers by interacting with similar or different types of receptors (56,86,259,260,261,262,263). Opioid receptors can exist as homodimers (263,264,265) or heteromers (263,266,267,268). Although dimers between the MOP and DOP receptors (266,267,269), DOP and KOP receptors (263) and MOP and NOP receptors were reported many years ago (270), dimers consisting of MOP and KOP receptors were initially a matter of dispute (86,263,265,271) before MOP-KOP receptor dimers were confirmed (272). Opioid receptor dimers were identified by crystal structures of opioid receptors *in vitro*, but the presence of these receptor-dimers in live cells have not been demonstrated yet

(14,15,16,17,18). It is therefore likely that the opioid receptor system also contains additional, so far unknown types of heterodimers, homodimers and/or oligomers.

1.2.2. Targeting of multiple opioid receptors to avoid opioid-induced adverse effects

Given the evidence for receptor heterodimers, it is likely that activation of one opioid receptor might affect the behaviour of another opioid receptor in the same complex. Different types of interactions are present between two or multiple receptors (61,262,273,274), which depend on the pharmacological profile of the ligands that interact with these receptors (275,276). The current literature suggests that most opioid receptor ligands are not extremely selective and could therefore bind to one/more off-target receptors to produce effects and adverse effects. For example, morphine at high doses can induce analgesia in MOP receptor knock-out mice by activating the KOP receptor (277). Similarly, in DOP receptor knockout mice a DOP-receptor agonist effectively produced analgesia, while a non-specific opioid antagonist (naltrexone) could reverse this effect (59). Although these data could be interpreted as non-specific interactions of the different DOP receptor agonists and antagonists, the authors interpreted it as evidence for the presence of a different DOP receptor subtype (59). However, recent studies suggest that opioid receptor subtypes may not actually exist, but these results rather reflect the presence of homo- or heteromeric receptor dimers (56). In light of a reduced DOP receptor activity in MOP receptor knock-out mice (61), it was hypothesised that the specific interaction between MOP and DOP receptors in specific neural pathways could modulate pain perception. In line with this hypothesis, co-administration of morphine and a DOP receptor antagonist induce analgesia, while surprisingly reducing tolerance in rodent models (122,123,124,125,278,279), which suggests that MOP and DOP receptor interactions regulate antinociceptive tolerance. It has to be noted, that under these conditions respiratory depression was not prevented, which suggests that additional receptor interactions are likely to

be involved (124).

These studies justify the approach to target two opioid receptors simultaneously, not only to explore the molecular mechanisms that contribute to tolerance but also to develop alternative drug candidates with reduced risk for the development of tolerance. The challenge ahead is to develop multi-target specific ligands that are effective as analgesics with a favourable side-effect profile. Several strategies for the simultaneous targeting of multiple receptors can be envisaged: (i) co-administration of two selective drugs, (ii) administration of one non-receptor-selective drug, or (iii) use of a single drug that specifically targets different receptors (i.e. multiple receptors selective ligand) (280). Especially the third strategy promises clinical advantages by reducing drug-drug interactions as well as pharmacokinetics and pharmacodynamics that will be easier to control (280,281,282).

Many new opioid receptor ligands have been designed to simultaneously target two or more opioid receptors and many of these are effective to produce an analgesic response *in vivo* (Table 3-5). Rational drug design and structure-activity relationship studies have evaluated the pharmacology of many of those ligands that act simultaneously on two different opioid receptors (282,283) or a combination of an opioid receptor with a non-opioid receptor (284,285,286). For example, the potent MOP-receptor agonists MDAN-21 is in fact not a selective MOP receptor agonist but rather a mixed MOP-receptor agonist/DOP-receptor antagonist that is 50-times more potent than morphine and produces less tolerance (287). This effect is likely the consequence of reduced internalisation of MOP-DOP receptor heterodimers due to a bridging of both receptors (288).

Studies showed that chronic administration of morphine increased MOP-DOP receptor heteromers in rostral ventral medulla in the brainstem, which is responsible for the processing

of nociceptive response (128,289). Different opioid ligands have been focussed to target the MOP-DOP receptor dimers with the objective that co-expression of MOP and DOP receptor might reduce analgesic tolerance (128,266,290,291). Some of these ligands showed selectivity for the MOP-DOP receptor dimer as well as the individual opioid MOP or DOP receptors (266), but other ligands do not show selectivity towards the individual receptors (291). MOP-DOP heteromer-biased ligands can activate both opioid-mediated and β-arrestin-mediated signalling. Anti-analgesic effect of DOP-MOP heteromers was also observed (292). The adverse effects profile of the heteromer-selective ligands is not clearly known (289), but the advancement of research related to opioids with selectivity for heteromers and/or individual receptors are increasing with time.

1.2.3. Barriers of characterising novel opioids

Currently, many new opioid ligands reported in the literature present with a mixed pharmacological profile in that they are activating and/or blocking different opioid receptor(s) (Table 3-5). Unfortunately, a detailed characterisation for some of these ligands with regards to their complete *in vitro* pharmacological profile is usually unavailable (Table 5). Mostly, the analgesic effects of these ligands and their partial *in vitro* profiles are reported to provide some limited information on their molecular activities. In general, the terms bifunctional, bivalent, dual or mixed opioids are widely used to describe these ligands without clear definitions for any of these terms (150,284,293,294,295,296). For example, MMP-2200 is a full agonist for the MOP and DOP receptor but the authors did not report its efficacy value (ED_{50}) towards the KOP receptor (Table 4), which makes it difficult to define the ligand as either a bifunctional, non-bifunctional or a biased agonist (151). Similarly, the ED_{50} value for the KOP receptor is not available for the ligands [Dmt1]DALDA, MGM-9, MGM-16 and AN-80 (Table 4) which makes the classification of these opioids as bifunctional, non-bifunctional or biased ligand

currently impossible (171,297,298,299,300). Therefore, the complete description of the *in vitro* pharmacological profiles of all new ligands should be envisaged as standard procedure to not only aide the molecular mechanism-based classification of these molecules but also as a prerequisite for the rational development of improved ligands with increased analgesic potential and a reduced liability for analgesic tolerance.

In vivo opioid receptor selectivity tests are routinely used to characterise novel ligands, which involve their co-administration with the selective antagonist for particular opioid receptors. One of the limitations of this approach is that potentially numerous interactions of a specific ligand with other relevant GPCRs in the nervous system are ignored. Given that these GPCRs have the potential to interact with opioid receptors, these interactions are likely to modulate test results. A better approach to test receptor selectivity *in vivo* is to use knockout animals for a particular opioid receptor, which should provide excellent proof of selectivity for new ligands. However, this approach is not without its own limitations, as knock out animals is known to display altered gene expression of unrelated genes, which could well affect the test results (301,302).It has to be acknowledged that only a combination of different approaches will realistically characterise the pharmacology of novel ligands compared to any of the individual *in vivo* or *in vitro* receptor selectivity assays that are currently widely employed.

1.2.4. Terminologies to characterise ligands targeting multiple receptors

Due to the obvious advantages to target multiple opioid receptors, the concept of bi-functional and bivalent opioids was developed. Ligands that contain two distinct pharmacophores to target two different opioid and/or non-opioid receptors are defined as bivalent. Previously, a distinction was made between bi-functional and bivalent ligands (282). It was proposed that bifunctional ligands can interact with two targets in a monovalent fashion (Fig. 1e, f). In contrast, bivalent ligands should contain two pharmacophores linked by a spacer to interact

with two different targets simultaneously such as within a receptor heterodimer (282) (Fig. 1a). However, bivalent ligands not necessarily bind selectively to two different specific receptors. For example, (-)(-) MCL-144, a MOP/KOP receptor bivalent ligand also shows a significant affinity towards the DOP receptor (303). Since ligands with two distinct pharmacophores can change their 3D confirmation under different physiological conditions, there is a chance of non-specific binding to other receptors or therapeutic targets (Fig. 1g-j). Thus, the term "bivalent" only explains the chemical structure of the compound, which does not describe their specific pharmacological properties. Therefore, to differentiate the effects of novel opioid analgesics based on their pharmacological activities, the terminology of "bifunctional analgesic" and "non-bifunctional analgesic" are used in this chapter.

Bi-functional opioid analgesics are compounds that (i) must produce antinociception, and (ii) must activate at least one and inhibit another opioid receptor; or (iii) activates several opioid receptors with a very high selectivity towards two specific receptors (Fig. 1a-f).

Non-bifunctional opioid analgesics are non-selective compounds that (i) also must produce antinociception, but (ii) have no specificity for any particular opioid receptor, with (iii) pharmacological data reported for at least three different opioid receptors.

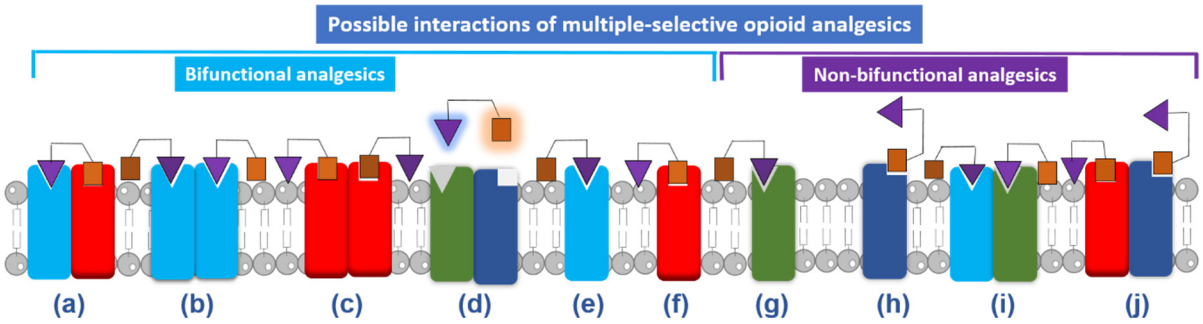


Figure 1. Possible *in vitro* interactions of opioid analgesics targeting multiple opioid receptors. The blue and red receptors are opioid receptors that are specific to the bifunctional ligand. Therefore the bifunctional ligands can interact with specific hetero-dimer (a), homodimer (b, c) or single opioid receptor (e, f), but do not interact with other opioid hetero-dimers (d). In contrast, non-bifunctional ligands can interact with all types of opioid receptors and heteromers (g, h, i, j).

Following the terminologies described in *section 1.2.4*, different OLs reported in the literature between the period of January 2008 and November 2017 are categorised in the following sections.

Table 3. *In vitro* characteristics of bifunctional opioid analgesic ligands. Keys: The values are presented as Mean ± SD. MOP: mu opioid receptor, DOP: delta opioid receptor, KOP: kappa opioid receptor, NOP: NO/FQ opioid receptor. i.p. intraperitoneal, s.c.: subcutaneous, '+' sign after a receptor symbol indicates an agonist, '-' sign after a receptor symbol indicates an antagonist, '-' sign before time means pre-administration. NLX: naloxone, NTX: naltrexone, NTL: naltrindole, nBNI: norBNI, Ki: affinity value, EC_{50}: effective dose 50%, Emax: maximum effect in percentage, Ke: antagonist affinity, pA_2 = antagonist affinity using Schild plot. n/a: not applicable/available, dns: does not stimulate, MDV: mouse vas difference, GPI: guinea pig ileum. The EC_{50} values were obtained from GTPγ^{35}S binding assays unless otherwise mentioned in parentheses.

Ligands	Selectivity	Parameters	MOP	DOP	KOP	NOP	Antinociception blocked by	Ref.
UMB-425	MOP+ DOP-	Ki, EC_{50}, Emax	3.2 ± 0.14 nM; 35 ± 3.7 nM; 73 ± 7.3%	208 ± 18 nM; dns; n/a (pA_2= - 0.91)	212 ± 21 nM; dns; n/a	n/a	NLX; Not by: nBNI	(150)
17d	MOP+ DOP- KOP-	Ki, EC_{50}, Emax	0.66 ± 0.06 nM; 1.74 ± 0.20 nM; 72 ± 2 %	1.20 ± 0.12 nM; dns; (Ke= 0.091 ± 0.01 nM)	1.82 ± 0.11 nM; dns; (Ke= 1.35 ± 0.28 nM)	n/a	n/a	(160)
SoRI-9409	MOP+ DOP-	Ki, EC_{50}, Emax	51.0 ± 8.0 nM; 163 ± 22 nM (GPI); n/a	2.2 ± 0.16 nM; N/A; 21% (MDV), (Ke = 0.66 nM)	20.0 ± 1.04 nM; n/a	n/a	β-FNA; Not by: NTL, nBNI	(155,304,305)
4 (VRP26)	MOP+ DOP-	Ki, EC_{50}, Emax	1.74 ± 0.2 nM; 36.9 ± 8.4 nM; 41.3 ± 2.8%	2.43 ± 0.34 nM; dns; (Ke= 6.1 ± 1.5 nM)	420 ± 25; dns;	n/a	NTX	(306)

Ligands	Selectivity	Parameters	MOP	DOP	KOP	NOP	Antinociception blocked by	Ref.
SR16435	NOP+ MOP+	Ki, EC_{50}, Emax	2.70 ± 0.1 nM, 2.73 ± 0.01 nM, 29.5 ± 10%	>1,000 nM, n/a	31.7 ± 4.8 nM, >10,000 nM, n/a	7.49 ± 1.31 nM, 28.7 ± 0.6 nM, 45.0 ± 5.1%	NLX, Potentiated by: SB612111; SR16430 (NOP-)	(152,307,308)
5a (AT-089)	NOP+ MOP+	Ki, EC_{50}, Emax	8.19 ± 0.16 nM, 56.9 ± 19.3 nM, 52.3 ± 6.7%	No affinity.	152 ± 25.7 nM, n/a (Ki > 100 nM)	3.16 ± 0 nM, 21.7 ± 5.6 nM, 96.5 ± 2.45%	NLX Potentiated by: SB612111	(309)
BU08028	MOP+ NOP+	Ki, EC_{50}, Emax	2.14 ± 0.79 nM, 6.03 ± 2.1 nM, 21.1 ± 8.7%	1.59 ± 0.28 nM, n/a*, 10.8 ± 6.8%	5.63 ± 1.30 nM, >10,000 nM	8.46 ± 1.31 nM, 78.6 ± 49 nM, 48 ± 13%	NLX; NTX; J-113397 (NOP-); Potentiated by SB612111	(152,310)
6 (UMB 246)####	MOP+ DOP-	Ki EC_{50} Emax	17.5 ± 1.1, 72 ± 11, 39 ± 1.3 %	14.4 ± 0.65, n/a, (Ke = 138 ± 24 nM)	1067 ± 39, n/a	n/a	n/a	(311)

**the stimulation on the DOP receptor was very low to accurately determine the EC_{50} value (152).*

Table 4. *In vitro* characteristics of non-bifunctional opioid analgesic ligands. Keys: The values are presented as Mean ± SD. MOP: mu opioid receptor, DOP: delta opioid receptor, KOP: kappa opioid receptor, NOP: NO/FQ opioid receptor, KO: knock-out, WT: wild-type. NTL: naltrindole, NTX naltrexone, nBNI: norBNI, Ki: affinity value, EC_{50}: Effective dose 50%, Emax: Maximum effect in percentage. n/a: not available. dns: does not stimulate, Ke: antagonist affinity. MDV: mouse vas difference, GPI: guinea pig ileum. The EC_{50} values were obtained from GTPγ^{35}S binding assays unless otherwise mentioned in parentheses.

Ligands:	**Selectivity *in vitro***	**Parameters**	**MOP**	**DOP**	**KOP**	**NOP**	**Antinociception blocked by**	**Ref.**
MMP-2200	MOP+ DOP+	Ki, EC_{50}, Emax	5.0 ± 0.65 nM, 220 ± 28 M, 85 ± 2.7 %	9.2 ± 1.7 nM, 14 ± 2.4 nM, 92 ± 2.3 %	42 ± 5.0 nM, n/a	n/a	β-FNA (Partial); NTL	(151)
[Dmt^{1}]DALDA	MOP+ KOP+	Ki, EC_{50}, Emax	0.143 ± 0.015 nM, 1.41 ± 0.29 nM (GPI), n/a	2100 ± 310 nM, 23.1 ± 2.0 nM (MDV), n/a	22.3 ± 4.2 nM, n/a	n/a	NLX	(297,298,299)
MGM-16	MOP+ DOP+	Ki, EC_{50}, Emax	2.1 ± 0.028 nM 18 ± 0.055 nM, 94 ± 1.8 %	7.0 ± 0.23 nM, 61 ± 3.2 nM, 105 ± 8.1 %	29 ± 1.5 nM, n/a	n/a	β-FNA; NTL (partial)	(300)
MGM-9	MOP+ KOP+	Ki, EC_{50}	7.3 ± 0.24 nM; n/a	350 ± 28 nM, n/a	18 ± 2.5 nM, n/a	n/a	NLX; β-FNA; norBNI; GNTI;	(171)
(-)(-) MCL144	MOP+ KOP+	Ki, EC_{50}, Emax	0.09 ± 0.004 nM; 1.3 ± 0.15 nM; 50 ± 6.6 %	4.2 ± 0.44 nM; n/a	0.05 ± 0.001 nM; 0.85 ± 0.053 nM; 60 ± 0.68 %	n/a	nBNI; β-FNA;	(303)

Ligands:	**Selectivity *in vitro***	**Parameters**	**MOP**	**DOP**	**KOP**	**NOP**	**Antinociception blocked by**	**Ref.**
AN81	MOP+ DOP+	Ki, EC_{50}, Emax	0.15 ± 0.02nM, 0.32 ± 0.0nM (GPI)	0.60 ± 0.07nM, 0.42 ± 0.02nM (MVD)	118 ± 12nM, n/a	n/a	n/a	(184)
1(4R)#	MOP+ DOP+ KOP+	Ki, EC_{50}, Emax	0.22 ± 0.02 nM; 1.6 ±0.3 nM; 81 ± 2 %	9.8 ± 0.8nM; 110 ± 6 nM; 16 ± 2 %	68 ± 2 nM; 540 ± 72 nM; 22 ± 2 %	n/a	NTX	(312)
14a	MOP+	Ki EC_{50} Emax	0.04, 0.93, 87%	0.02, dns	48, dns	n/a	NTX	(313)
16 (MP1104)	MOP+ DOP+ KOP+	Ki EC_{50} Emax	0.021 ± 0.003, 0.21 ± 0.03, 103 ± 2.5 %	0.08 ± 0.019, 0.41 ± 0.11, 88 ± 0.38 %	0.006 ± 0.002, 0.027 ± 0.002, 104 ± 2.3 %	n/a	nBNI, NTL; KOP-KO mice	(314)
10j	MOP+ KOP+	Ki EC_{50} Emax	0.15 ± 0.08, 3 ± 1, 96 ± 4 %	15 ± 5, dns	2 ± 1 15 ± 9, 14 ± 2	n/a	n/a	(315)
4h	MOP+ DOP+	Ki EC_{50} Emax	0.19, 0.78, 95 %	0.51, 14, 40 %	29, 250, 28 %	n/a	n/a	(295)
13a (KGNOP1)##	MOP+ DOP+ KOP+ NOP-	Ki EC_{50}	5.0 ± 1.7, 6.1 ± 0.1 (GPI)	99 ±4, 5.3 ± 1.4 (MDV)	33 ± 15, n/a	42 ± 6, >10000	n/a	(316)

Ligands:	**Selectivity *in vitro***	**Parameters**	**MOP**	**DOP**	**KOP**	**NOP**	**Antinociception blocked by**	**Ref.**
4	MOP+ DOP+ KOP+	pIC_{50} pEC_{50}	0.25 ± 0.02, 8.25,	51.20 ± 3.00, 7.19,	1.78 ± 0.15, 7.38,	n/a	n/a	(317)
11	MOP+ DOP+ KOP+	pIC_{50} pEC_{50}	0.49 ± 0.06, 8.78	11.2 ± 5.2, 8.06	0.30 ± 0.05, 8.87	n/a	β-FNA	(318)
DeNo	MOP+ DOP+ KOP+ NOP+	Ki pEC_{50}	9.55 ± 0.10, 7.77	8.12 ± 0.11, 6.78	7.34 ± 0.13 5.92	10.2 ± 0.09, 9.5	n/a	(319)
BN-9[###]	MOP+ DOP+ KOP+	pEC_{50} Emax	6.51±0.15 92.4±9.7%	6.23±0.06 82.2±3.7%	6.25±0.25 105.6±20.7%	n/a	NLX; nBNI; β-FNA	(178)
PPL-101	MOP+ DOP+ KOP+	Ki EC_{50} Emax	0.35 ± 0.04 0.3 ± 0.1 12 ± 2.9	4.0 ± 1.4 40 ± 6.3 22 ± 5.8	0.43 ± 0.1 15 ± 2.5 63 ± 0.3	n/a	JDTic; Not by: β-FNA	(320)

[#]1(4R): a peptide, poor bioavailability (306).

[##]13a is an antagonist for NOP receptor (pA_2, 6.00 ± 0.05), which is inactive after oral administration.

[###]BN-9: data from cAMP assay (178)

Table 5. *In vitro* characteristics of opioid analgesic ligands with the incomplete pharmacological profile. Keys: The values are presented as Mean ± SD. MOP: mu opioid receptor, DOP: delta opioid receptor, KOP: kappa opioid receptor, NOP: NO/FQ opioid receptor, KO: knock-out, WT: wild-type. NTL: naltrindole, NTX naltrexone, nBNI: norBNI, Ki: affinity value, EC_{50} : Effective dose 50%, Emax: Maximum effect in percentage. n/a: not available, dns: does not stimulate, Ke antagonist affinity. MDV: mouse vas difference, GPI: guinea pig ileum. The EC_{50} values were obtained from GTPγ^{35}S binding assays unless otherwise mentioned in parentheses.

Ligands:	**Selectivity *in vitro***	**Parameters**	**MOP**	**DOP**	**KOP**	**NOP**	**Antinociception blocked by**	**Ref.**
10	MOP+ DOP+	Ki, EC_{50}, Emax	0.48 ± 0.06 nM, 13 ± 1.1nM, 47 ± 4 % (GPI IC_{50}= 2.5 ± 0.7 nM)	0.65 ± 0.35 nM, 44 ± 5.9 nM, 56 ± 6 % (MVD IC_{50} = 9.3 ± 0.4 nM)	n/a	n/a	n/a	(321)
RV-Jim-C3	MOP+ DOP+	Ki	n/a	n/a	n/a	n/a	NTL, CTAP	(322)
1	MOP+ DOP+	Ki, EC_{50}, Emax	1.1 nM, 50 ± 6.5 nM (GPI),	0.72 nM, 33 ± 9.4 nM (MDV),	n/a	n/a	n/a	(323)
SR14150[#]	NOP+ MOP+	Ki, EC_{50}, Emax	29.9 ± 2.1 nM; 99 ± 12 nM; 23.4 ± 3.2 %	n/a	42.7 ± 1.0 nM, 276 ± 75.8 nM, 38 %	1.39 ± 0.42 nM, 20.8 ± 3.1, 54.2 ± 10.9 %	NLX	(283,308)

Ligands:	**Selectivity *in vitro***	**Parameters**	**MOP**	**DOP**	**KOP**	**NOP**	**Antinociception blocked by**	**Ref.**
SR16507[##]	NOP+ MOP+	Ki, EC_{50}, Emax	1.07 ± 0.17 nM, 5.2 ± 1.6 nM, 47 ± 1.5 %	n/a	82.4 ± 16.4 nM,	5.22 ± 0.65 nM, 8.5 ± 0.8 nM, 95 ± 12 %	NLX	(283,307)
RSA 504	MOP+ DOP+	Ki, EC_{50}, Emax	27 nM , 0.47 nM, 110 % (GPI: 210 ± 52 nM)	23 nM, 5.5 nM, 81 % (MDV: 23 ± 9.7 nM)	n/a	n/a	n/a	(284) (294)
RSA 601	MOP+ DOP+	Ki, EC_{50}	5.7 nM, 71 nM (GPI)	0.55 nM, 24 nM (MVD)	n/a	n/a	n/a	(294) (324)
TY027	MOP+ DOP+	Ki, EC_{50}, Emax	16 nM, 7.0 nM, 55 % (GPI IC_{50}: 490 ± 29 nM)	0.66 nM, 8.6 nM, 58 % (MDV IC_{50}: 15 ± 2.0 nM)	n/a	n/a	n/a	(325,326)
TY005	MOP+ DOP+	Ki, EC_{50}, Emax	36 nM, 32 nM, 42 ± 2 % (GPI IC_{50}: 360 ± 130 nM)	2.8 nM, 2.9 nM, 45 ± 3 % (MDV IC_{50}: 22 ± 1.2 nM)	n/a	n/a	β-FNA; NTL	(327,328)

Ligands:	**Selectivity *in vitro***	**Parameters**	**MOP**	**DOP**	**KOP**	**NOP**	**Antinociception blocked by**	**Ref.**
Tapentadol ###	MOP+	Ki	96 ± 9 nM	970 ± 10 nM	910 ± 90 nM	n/a	NLX	(329)
13	MOP+ DOP+	Ki EC_{50}	1.82, 13.0 (GPI)	11.2, 12.6 (MDV)	n/a	n/a	n/a	(330)
5 (AKG127)	MOP+ DOP+	Ki EC_{50}	1, 42 ± 9.7 (GPI), 59 ± 23 (cAMP)	1, 2 ± 0.68 (MDV) 21 ± 14 (cAMP)	n/a	n/a	n/a	(331)
26	MOP+ DOP+ KOP+	Ki	8 ± 2.3	0.7 ± 0.3	67 ± 23	n/a	NTI (WT); MOP-KO, KOP-KO mice	(332)
22	MOP+ DOP+	Ki EC_{50}	0.08, 1.86 (GPI)	0.28, 2.16 (MDV)	n/a	n/a	n/a	(293)
11a	MOP+ KOP+	pIC_{50}	1.61	n/a	2.73	n/a	n/a	(333)
6 (UMB 246)	MOP+ DOP-	Ki EC_{50} Emax	17.5 ± 1.1, 72 ± 11, 39 ± 1.3 %	14.4 ± 0.65 n/a	1067 ± 39 n/a	n/a	n/a	(311)

#SR14150 (30 mg/kg s.c.): Animals were not moving and tried to stay curled up at 30 min post-injection (283). Values are shown as, Mean ± SD.

##SR16507 (3 mg/kg s.c.): Animals treated with SR16507 were preferred to be still (283). Values are shown as Mean ± SD.

###Tapentadol: Data was obtained from rat opioid binding assay (329)

1.2.5. Bifunctional opioid analgesics

1.2.5.1. Pharmacological profiles of bi-functional opioid analgesics

Over the period of January 2008 to November 2017, eight ligands with multiple selectivity and complete *in vitro* pharmacological profile for at least three of the major opioid receptors (MOP, DOP, KOP) have been described (UMB-425, 17d, SoRI-9404, VRP26, SR16435, 5a, UMB 246 and BU08028) (150,152,160,304,307,309,310), with three of these (SR16435, 5a and BU08028) also showing NOP receptor affinity (Table 3). All of these ligands showed better MOP selectivity than towards other ORs. In addition, five of these compounds acted as DOP receptor antagonist, while three other ligands did not show any selectivity for this receptor. In addition to their agonistic effects towards the MOP receptor, SR16435, AT-089 and BU08028 showed similar agonism towards the NOP receptor. Overall, UMB-425, 17d, SoRI-9409, UMB 246 and VRP26 are MOP receptor agonists and at the same time DOP receptor antagonists, while SR16435, AT-089, BU08028are MOP and NOP receptor antagonists, without affecting the DOP and KOP receptors (Table 3). Apart from their *in vitro* characterisation, these bifunctional compounds were mostly tested for their antinociceptive effects in mice to investigate the capacity of these ligands to penetrate to the central nervous system.

1.2.5.2. Antinociceptive effects of bifunctional opioid analgesics

Among the eight reported bi-functional ligands, six (UMB-425, 17d, SoRI-9409, SR16435, 5a and BU08028) were tested for antinociception in male mice, sex-related information was missing for animals tested with UMB 246, while VRP26 was tested using both male and female mice (Table 6). This is a significant experimental limitation as sex-specific differential opioid effects have been reported for rodents (334,335), and female animals have been used comparatively less than males to avoid the interference of hormonal effects (336). Since most of these OLs were not tested in female animals, differential gender-specific antinociceptive

effects are so far unknown for these compounds. Overall, antinociception induced by these OLs was tested using tail-flick test by radiant heat or warm water (50-55 ºC) tail-withdrawal assay with cut-off times ranging from 10-20 seconds, whereas baseline latency was approximately 5 seconds (Table 6). Apart from experimental variations, all antinociception results were consistently expressed as *Maximum Possible Effect* (MPE) based on their respective latencies, which allows a superficial level of comparison. The reported MPE varied between 40 - 100 % for these ligands at the maximal tested dose, while peak antinociception ranged between 30 - 120 min post administration times. Antinociception (median effective doses (ED_{50} or MPEs) compared to morphine were reported for six of these ligands (UMB-425, 17d, SoRI-9409, 5a, VRP26 and BU08028), while SR16435 and UMB-246 were not compared to morphine (Table 6).

Table 6. Antinociceptive effects of bifunctional opioid analgesic ligands using tail flick assay. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, other values are Mean ± SEM (standard error mean), CL: cut-off latency, SW: Swiss Webster, SD: Sprague Dawley, WWTW: warm water tail withdrawal assay, TF: tail flick assay by radiant heat, TFL: tail-flick latency, FEN: fentanyl, s.c.: subcutaneous, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, '-' sign before time means pre-administration. n/a: not applicable/available.

Ligands (route of administration)	Animal, assay, Cut-off Latency, baseline latency, %MPE	%MPE of Ligand (dose, post-admin time)	%MPE of Morphine (or other) (dose, post-admin time)	ED_{50} of Ligand	ED_{50} of Morphine (or other)	Ref.
Tail flick (TF) / Warm water tail withdrawal (WWTW) assay						
UMB-425 (s.c.)	Male SW mice (21-30g), TF, 10s, 2-4s, %CL	~90% (20 mg/kg, 30 min)	~100% (20 mg/kg, 30 min)	8.83 mg/kg	6.85 mg/kg	(150)
17d (i.c.v.)	Male ICR mice, WWTW 55°C, 10s, no BL, %CL	75% (1 nmol, 45 min)	100% (10 nmol)	0.35 nmol	0.43 nmol	(160)
SoRI-9409 (i.c.v.)	Male ICR mice (20-30g), WWTW 55°C, 15s, ≤5s, %CL	40.3% (100 nmol, 20 min)	~90% (10 nmol, 30 min)	> 100 nmol	2.94 nmol	(155,304)
4 (VRP26) (i.p.)	Male & female C57BL/6 mice (20-35g), WWTW 50°C, 20s, 3-6s, %CL	~100% (20s) (32 mg/kg, 45 min)	~100% (10 mg/kg, 30 min) (312)	n/a	n/a	(306)

Ligands (route of administration)	**Animal, assay, Cut-off Latency, baseline latency, %MPE**	**%MPE of Ligand (dose, post-admin time)**	**%MPE of Morphine (or other) (dose, post-admin time)**	**ED_{50} of Ligand**	**ED_{50} of Morphine (or other)**	**Ref.**
VRP26 (i.p.; s.c.)	Male & female C57BL/6 mice (20-35g), WWTW 50°C, 20s, ~5s, %CL	~100% (20s, 10 mg/kg, 30 min)	FEN:~100% (0.3 mg/kg, 30 min)	5.44±0.10 mg/kg (i.p.); 5.08±0.23 mg/kg (s.c.)	FEN: 0.17±0.013 mg/kg (i.p.); 0.20±0.001 mg/kg (s.c.)	(337)
SR16435 (s.c.)	Male ICR mice (25-30g), TF, 15s, 5.03±0.3s, %CL	~90% (30 mg/kg, 30 min)	n/a	n/a	n/a	(307)
5a (s.c.)	Male ICR mice (25-30g), TF, 15s, 5.5±0.16s, %CL	~40% (10 mg/kg s.c., 60 min)	~100% (10 mg/kg s.c., 60 min)	n/a	n/a	(309)
BU08028 (s.c.)	Male ICR mice (25-30g), TF, 15s, 4.32±0.12s, %CL	~95% (30 mg/kg, 120 min) ~90% (10 mg/kg, 240 min)	~100% (10 mg/kg, 30 min)	n/a	n/a	(152)
BU08028* (s.c.)	Male and female rhesus monkeys (10-17 y), WWTW 50°C, 20s,	~100% (0.01 mg/kg, 1-24 h)	n/a	n/a	n/a	(310)
UMB 246	Mice, no info	~60% (60 mg/kg s.c., 30 min)	n/a	n/a	n/a	(311)

**BU08028: Animals were reported with no reinforcing effects and no respiratory depression (310)*

Apart from the universally used tail flick assay, antinociception by UMB-425 and UMB-246 was also quantified using the hot plate assay, while SR16435 prevented mechanical allodynia, and SoRI-9404 produced antinociception in an acetic acid-induced inflammation pain assay. Although the ED_{50} values of UMB-425 and SoRI-9404 in the hot plate or inflammation pain assay were higher than morphine, their MPE values were similar to morphine at the highest dose tested (Table 7). Overall, seven ligands produced relatively similar antinociception levels to morphine after acute administration, while UMB-246 was not directly compared against any other OL.

Table 7. Effects of bifunctional opioid analgesic ligands using different antinociception tests. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, other values are Mean ± SEM (standard error mean), CL: cut-off latency, SW: Swiss Webster, SD: Sprague Dawley, HP: hot plate, FEN: fentanyl, s.c.: subcutaneous, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, i.t.: intrathecal, AAW: acetic acid writhing. n/a: not applicable/available.

Ligands	**Animal, assay, Cut-off Latency, baseline latency, %MPE**	**%MPE of Ligand (dose, post-admin time)**	**%MPE of Morphine (dose, post-admin time)**	**ED_{50} of Ligand**	**ED_{50} of Morphine**	**Ref.**
		Hot-plate (HP) assay				
UMB-425	Male SW mice, HP (53°C), 30s, 8-10s, %CL	~90% (20mg/kg s.c., 30 min); ~100% (10mg/kg s.c., 50 min)	~100% (20mg/kg s.c., 30 min)	4.30 mg/kg s.c. (30 min)	2.73 mg/kg s.c. (30 min)	(150)
UMB-246	Mice	~40% (60 mg/kg s.c., 50 min)	n/a	n/a	n/a	(311)
		Mechanical allodynia test				
Ligands:	**Animal (SNL), baseline threshold**	**Peak threshold of Ligand (dose, post-admin time)**	**Peak threshold of Morphine (dose, post-admin time)**	**ED_{50} of Ligand**	**ED_{50} of Morphine**	**Ref.**
SR16435	Male ICR mice (25–30 g), 0.09± 0.01 g	92±6% (~1.8g) (3µg i.t., 30 min)	89±6% (~1.7g) (10µg i.t., 30 min); (SCH221510: 91±2%, ~1.9 g; 10µg i.t., 30 min)	n/a	n/a	(338)
SR16435	Male SD rats (250-300g), 4.3 ± 0.9 g	~12 g (10 mg/kg i.p., 60 min)	~10 g (10 mg/kg i.p., 60 min)	n/a	n/a	(308)

Acetic acid writhing assay						
Ligands:	**Animal, assay, %MPE**	**%MPE of Ligand (route of administration)**	**%MPE of Morphine (route of administration)**	**ED_{50} of Ligand**	**ED_{50} of Morphine**	**Ref.**
SoRI-9409	Male ICR mice (20-30 g), %MPE	~80% (i.c.v.) ~100% (i.p.)	~90% (i.c.v.) ~100% (i.p.)	7.5 nmol (i.c.v) 11.5 mg/kg (i.p.)	0.004 nmol (i.c.v.) 2.3 mg/kg (i.p.)	(155)

Similar to the *in vitro* selectivity tests, the involvement of specific OR for the antinociceptive effects was also investigated using co-administration of non-selective (naloxone) or selective OR antagonists (β-FNA, naltrindole, nor-BNI for MOP, DOP and KOP receptors respectively) *in vivo*. Overall the receptor selectivity of the eight bifunctional ligands for antinociceptive effects *in vivo* reflected their *in vitro* receptor selectivity and thus confirmed the involvement of specific opioid receptors for each ligand that are responsible for their antinociceptive effects and likely also their effect on tolerance (Table 3). To investigate whether these bifunctional OLs can avoid antinociceptive tolerance that is a major drawback of all clinically used opioids, their efficacy over several days of repeated administration has been typically compared against morphine or fentanyl.

1.2.5.3. Effects of bi-functional opioid analgesics on antinociceptive tolerance

Previous studies suggest that simultaneous activation of the MOP receptor and inhibition of the DOP receptor can prevent tolerance. One of the first studies reported that coadministration of morphine with the DOP receptor antagonist naltrindole can reduce morphine tolerance (122). This hypothesis was supported by the observation that DOP receptor knockdown (339) and DOP receptor knockout mice (59) showed reduced levels of drug-induced tolerance. In the later study, daily administration of morphine over 5 days produced tolerance in wild-type animals, whereas no tolerance was observed over 8 days in DOP receptor knockout animals (59). These studies strongly support the hypothesis that targeting multiple OR, especially the MOP and DOP receptors, has the potential to reduce antinociceptive tolerance.

Out of eight bifunctional opioid analgesics (Table 3), antinociceptive tolerance was tested for only five ligands (Table 8), using repeated administration of 1 to 3 doses daily over a period of 3 to 9 days. Antinociceptive effects were measured as reduction of MPE (%) or fold-shifts of antinociceptive ED_{50}. All five ligands were compared with morphine or fentanyl at their

equivalent analgesic doses. Thus, differences compared to morphine or fentanyl provided insight towards understanding the potency of these ligands. Four ligands (UMB-425, 17d, VRP26 and BU08028) were tested for tolerance by measuring spinal antinociception (e.g. tail-flick test, warm-water tail withdrawal assay), while for SoRI-9409 the inflammatory antinociception assay (AAW) was employed. Overall, MOP receptor agonist / DOP receptor antagonist ligands showed less tolerance, while the MOP / NOP receptor agonist BU08028 induced slightly more tolerance than morphine (Table 8). However, BU08028 showed fewer adverse-effects (less respiratory depression and dependence) than morphine in primates (310). Therefore, bifunctional opioid analgesics showed overall a trend towards reduced antinociceptive tolerance and/or other adverse effects.

Table 8. The effects of bifunctional opioid analgesic ligands on sub-chronic administration and antinociceptive tolerance. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, WWTW: warm water tail withdrawal assay, TF: tail flick assay by radiant heat, HP: hot plate, FEN: fentanyl, s.c.: subcutaneous, i.p.: intraperitoneal. AAW: acetic acid writhing. n/a: not applicable/available. MOR: morphine, b.i.d.: twice daily, t.i.d.: three times daily. Note: Same experimental conditions/animals apply for the TF or WWTW assay with acute administration.

Ligand	Procedure summary	Result	%MPE of ligand	% MPE of morphine	Tolerance ED_{50} shift of Ligand	Tolerance ED_{50} shift of Morphine	Ref.
UMB-425	UMB-425 (15 mg/kg s.c.) or MOR (15 mg/kg s.c.) were administered twice daily for 5 days. Antinociception was assessed in tail flick and hot plate assays at 30 min post admin time. Tolerance ED_{50} was measured on Day 6.	Reduced tolerance: Less ED_{50} shifts than MOR	Day 1: ~100 % (both) Day 4: ~85% (HP) ~90% (TF)	Day 1: ~100% (both) Day 4: ~60% (HP) ~75% (TF)	TF: 1.3 fold HP: 3 fold	TF: 6.4 fold HP: 7.8 fold	(150)
17d	17d and MOR were administered at their ED_{90} dose twice daily for 3 days. The tolerance ED_{50} was determined on Day 4 using WWTW (55°C) assay. (Note: ED_{50} of 17d: 0.35 nmol and MOR: 0.43 nmol)	Reduced tolerance: Less ED_{50} shift than MOR	Day 1: ~75%	Day 1: ~100%	7.9 fold	44 fold	(160)

Ligand	Procedure summary	Result	%MPE of ligand	% MPE of morphine	Tolerance ED_{50} shift of Ligand	Tolerance ED_{50} shift of Morphine	Ref.
SoRI-9409	Antinociception was measured in AAW at ED_{90} doses of morphine (6mg/kg i.p.) and SoRI-9409 (30mg/kg i.p.) twice or thrice daily for 3 days. Tolerance ED_{50} was measured on Day 4.	Reduced tolerance: Less ED_{50} shift than MOR	Day 1: ~80% Day 4: ~85% (b.i.d.) ~70% (t.i.d.)	Day 1: ~95% Day 4: ~ ~90% (b.i.d.) ~85% (t.i.d.)	0.6 fold (b.i.d.) 1.1 fold (t.i.d.)	2.4 fold (b.i.d.) 5.1 fold (t.i.d.)	(155)
VRP26	FEN (0.3 mg/kg s.c) or VRP 26 (10 mg/kg s.c.) were administered daily continuously via osmotic pumps for 7 days. Tolerance ED_{50} was measured on Day 8 in WWTW (50°C assay)	Reduced tolerance: Less ED_{50} shift than FEN. No difference in latencies on Day 1 and Day 8.	Latency: Day 1 ~20 s Day 8: ~20 s	Latency: FEN: Day 1 ~20 s Day 8: ~20 s	No shift	FEN: 3 fold	(337)
BU08028	MOR (10 mg/kg s.c.) and BU08028 (10 mg/kg s.c.) were injected once daily for 9 days and tested on days 1,2,4 and 9 at 1-hour post injection in TF assay.	Morphine tolerance: Day 9 BU08028 tolerance: Day 4	Day 1: ~85% Day 4: ~50% Day 9: ~15%	Day 1: ~90% Day 4: ~70% Day 9: ~15%	n/a	n/a	(152)

1.2.6. Non-bifunctional opioid analgesics

1.2.6.1. Pharmacological profiles of non-bifunctional ligands

In addition to bifunctional OLs, several non-bifunctional OLs with antinociceptive effects have been reported over the same time-period and were categorised following our terminologies. Noticeably, all of these ligands are MOP, DOP and KOP receptor agonists (Table 4), but their selectivity profiles towards different ORs were similar and not specific to any particular OR. For eleven of these OLs, the specific opioid receptors responsible for their antinociceptive effects were also identified by co-administration of a non-selective or selective antagonist of the MOP, DOP and KOP receptors (Table 4). In addition, antinociception induced by ligand 16 (MP1104) reportedly involve the KOP receptor as its antinociceptive effect was abolished in KOP receptor knock out mice (314). To my knowledge, similar studies have not been reported for other sixteen OLs, which is a source of uncertainty regarding the actual mode of action of these ligands (Table 4).

1.2.6.2. Antinociceptive effects of non-bifunctional ligands

The antinociception characteristics for these non-bifunctional OLs were assessed by either using thermal (e.g. tail-flick; hot plate) or mechanical (e.g. von Frey filament) assays in mice or rats (Table 9, 10 and 11). From these, fourteen ligands (82 %) were reported for antinociceptive effects using tail flick assay (Table 9). Noticeably, only BN-9 was tested using three different routes of administration, another three ligands (AN81, MGM-9 and MGM-16) were tested using two different routes of administration and the rest of the compounds were tested using a single route of administration. Subcutaneous (5), intraperitoneal (4) or intravenous (3) were commonly used routes of administration in these preclinical studies. Noticeably, only two ligands (MGM-9 and MGM-16) produced antinociceptive response, when the compounds were administered orally (Table 9). There are variations in the

experimental settings of tail-flick assay, such as cut-off latency was varied from 7 to 20 seconds with a baseline latency was used between 2 to 6 seconds (Table 9). In most cases, antinociception was determined as MPE (%). Antinociception (MPE) produced by eight (57%) of these ligands were similar to morphine (Table 9).

Table 9. The antinociceptive effects of non-bifunctional analgesic ligands using tail flick assay. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, other values are Mean (± SEM (standard error mean)), CL: cut-off latency, SW: Swiss Webster, SD: Sprague Dawley, WWTW: warm water tail withdrawal assay, TF: tail flick assay by radiant heat, HP: hot plate, TFL: tail flick latency, FEN: fentanyl, MOR: morphine, s.c.: subcutaneous, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, i.t.: intrathecal, admin. route: route of administration, n/a: not applicable/available, BL: baseline latency.

Ligands (admin. route)	**Animal, assay, Cut-off latency, baseline latency, %MPE**	**%MPE of Ligand (dose, post-admin time)**	**%MPE of Morphine (dose, post-admin time)**	**ED_{50} of Ligand**	**ED_{50} of Morphine**	**Ref.**
MMP-2200 (s.c.)	Male ICR mice (25-35 g), WWTW 55° C, 10s, <5s, %CL	~90% (20 mg/kg, 30 min)	~100% (20 mg/kg, 30 min)	8.90 µmol/kg	23.80 µmol/kg	(151)
MGM-16 (s.c.)	Male ddY mice, TF, 10s, no BL, %CL	~95% (0.2 mg/kg, 15 min)	n/a	0.064 mg/kg	4.57 mg/kg	(300)
MGM-16 (p.o.)	Male ddY mice, TF, 10s, no BL, %CL	~98% (1 mg/kg, 15 min)	n/a	0.263 mg/kg	63.0 mg/kg	(300)
AN81 (i.v.)	Male C57Bl6 mice (25-28 g) 10 s, WWTW 55° C, 10s, %CL	100% (4 mg/kg, 30 min) 95 ± 2.89% (1 mg/kg, 30 min)	95.08 ± 1.67% (4 mg/kg, 15 min)	n/a	n/a	(184)
AN81 (s.c.)	Male CD1 mice (27-33 g), TF, 10 s, no info, %CL	~85% (1 mg/kg, 60 min)	~95% (5 mg/kg, 30 min)	n/a	n/a	(184)

Ligands (admin. route)	Animal, assay, Cut-off latency, baseline latency, %MPE	%MPE of Ligand (dose, post-admin time)	%MPE of Morphine (dose, post-admin time)	ED_{50} of Ligand	ED_{50} of Morphine	Ref.
1(4R) (i.p.)	Male C57BL/6 mice (20-30g),WWTW 50° C, 20s, 3-6s, %CL	100% (10 mg/kg, 15 min)	100% (10 mg/kg, 30 min)	3 mg/kg	n/a	(312)
(-)(-)MCL144 (icv.)	Male, ICR mice (20–30 g), WWTW 55°C, 15s, <5s, %CL	95% (10 nmol, 20 min)	n/a	30 nmol	n/a	(303)
MGM-9 (s.c.)	Male ddY mice(25-33 g), TF, 10s, no BL, %CL	95% (2 mg/kg, 15 min)	90% (8mg/kg, 30 min)	0.57 mg/kg	4.57 mg/kg	(171)
MGM-9 (p.o.)	Male ddY mice (25-33 g), TF, 10s, no BL, %CL	85% (8mg/kg, 15 min)	75% (100 mg/kg, 60 min)	2.84 mg/kg	63.0 mg/kg	(171)
[Dmt^1]DALDA (i.t.)	Male SD rats (300-350g), TF, 7s, 2.5-3.5s, %CL	~100% (7s) (3.18 pmol, 60 min) (~3000 times more potent than morphine)	~93% (~6.5s) (9990 pmol, 60 min)	1.06 pmol	3330 pmol	(298)
14a (i.p.)	Male C57BL/6 mice (20-30g), WWTW, 50° C, 20s, 3-6s, latency	~20s (10 mg/kg)	n/a	4.73±0.08 mg/kg	n/a	(313)

Ligands (admin. route)	Animal, assay, Cut-off latency, baseline latency, %MPE	%MPE of Ligand (dose, post-admin time)	%MPE of Morphine (dose, post-admin time)	ED_{50} of Ligand	ED_{50} of Morphine	Ref.
16 (MP1104) (s.c.)	Male CD1 mice (20-32 g), TF, 10 s, 2-3s, %CL	~95% (1 mg/kg, 30 min)	n/a	0.33±0.09 mg/kg	n/a	(314)
10j (i.p.)	Male C57BL/6 mice (20-30 g), WWTW, 50° C, 20s, 3-6s, latency	20s (10 mg/kg, 30 min)	n/a	n/a	n/a	(315)
4h (i.p.)	Male C57BL/6 mice (20-30 g), WWTW 50° C, 20s, ~5s, latency	~20 s (10 mg/kg, 30 min)	n/a	n/a	n/a	(295)
13a (i.v.)	MaleC57BL6 (20-25 g), TF, 10s	100% (31 nmol, 60 min)	100% (390 nmol, 30 min)	n/a	n/a	(316)
BN-9 (i.c.v.)	Male Kunming mice, TF, 10s, 3-5s	~95% (2 nmol, 15 min)	~90% (4nmol, 30 min)	0.39 nmol	1.02 nmol	(178)
BN-9 (i.t.)	Male Kunming mice, TF, 10s, 3-5s	~100% (2 nmol, 10 min)	~95% (4nmol, 10 min)	0.29 nmol	0.35 nmol	(178)
BN-9 (i.v.)	Male Kunming mice, TF, 10s, 3-5s	~90% (224 nmol)	~90%	58.6 nmol	46.2 nmol	(178)

Ligands (admin. route)	Animal, assay, Cut-off latency, baseline latency, %MPE	%MPE of Ligand (dose, post-admin time)	%MPE of Morphine (dose, post-admin time)	ED_{50} of Ligand	ED_{50} of Morphine	Ref.
PPL-101	Male ICR mice, TF, 15s, 4.83s	~90% (3 mg/kg, 60 min)	~100% (15 mg/kg)	n/a	n/a	(320)
KGNOP1 (13a)	Male Wister rats (250-350 g), TF, 9s	~100% (5 nmol, i.t., 30 min)	~85% (3.5 nmol, i.t., 30 min)	n/a	n/a	(340)

Apart from using the tail-flick assay, four non-bifunctional OLs were tested in male mice for their antinociceptive effects using the hot plate test. While the non-bifunctional ligands 4 and 11 showed 80 to 100 % MPE, ligand 13a showed only 30 % MPE (Table 10). Noticeably, the cut-off latency used in these studies varied between 25 to 240 seconds, which indicate very significant variations with regards to the antinociceptive responses of the ligands (Table 10).

Table 10. Antinociceptive effects of non-bifunctional analgesic ligands using hot plate assay. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, other values are Mean (± SEM (standard error mean)), CL: cut-off latency, HP: hot plate, FEN: fentanyl, s.c.: subcutaneous, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, n/a: not applicable/available.

Ligands (admin route)	Animal, assay, Cut-off latency, baseline latency, %MPE	%MPE of Ligand (dose, post admin time)	%MPE of Morphine (dose, post admin time)	ED_{50} of Ligand	ED_{50} of Morphine	Ref.
MGM-9 (s.c.)	Male ddY mice (25-33 g), 55±0.2° C, 30s, no info, %CL	~95% (2.0mg/kg, 7.5 min)	~90% (8mg/kg, 30 min)	0.70 mg/kg	4.08 mg/kg	(171)
MGM-9 (p.o.)	Male ddY mice (25-33 g), 55±0.2° C, 30s, no info, %CL	~90% (8mg/kg, 7.5 min)	~80% (100mg/kg, 30 min)	2.98 mg/kg	48.2 mg/kg	(171)
4 (i.c.v.)	Male Albino mice (20-26 g), 55±0.5° C, 240s, no info, %CL	~100% (0.1µg, 10µl, 10 min)	n/a	0.06µg	n/a	(317)
4 (i.p.)	Male Albino mice (20-26 g), 55±0.5° C, 240s, no info, %CL	82% (10 mg/kg, 15 min)	n/a	1.160 mg/kg	n/a	(317)
11 (i.c.v.)	Male albino (CD1) mice (20-26 g), 55±0.5° C, 240 s, no info, %CL	~90% (0.1µg, 10µl, 10 min)	n/a	n/a	n/a	(318)
11 (i.p.)	Male albino (CD1) mice (20-26 g), 55±0.5° C, 240s, no info, %CL	~84% (10 mg/kg, 15 min)	n/a	n/a	n/a	(318)
KGNOP1 (13a)	Male SD rats (250-350 g), 52±0.2° C, 60s, %CL	~30% (0.34 µmol/kg, 180 min)	~90% (17.52 µmol/kg, 180 min)	0.41 µmol/kg	14.7 µmol/kg	(341)

The antinociception of four non-bifunctional ligands was further tested using spinal nerve ligated animals. In these studies, antinociceptive latency or threshold were measured using Hargreaves' method or von-Frey filaments respectively (Table 11), which assess peripheral antinociception (342). In this setting, [Dmt^{1}]DALDA produced paw withdrawal latencies and mechanical allodynia comparable to morphine, while similar effects were reported with 13a and DeNo using mechanical allodynia. Therefore, these non-bifunctional OLs appear to be effective against neuropathic pain (Table 11).

Table 11. Antinociceptive effects of non-bifunctional opioid analgesic ligands using paw withdrawal latency and paw withdrawal threshold assays. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50 %, '~' shows an approximate value, other values are Mean (± SEM (standard error mean)), SD: Sprague Dawley, s.c.: subcutaneous, i.t.: intrathecal. n/a: not applicable/available. SNL: spinal nerve ligated, CPIP: Chronic Postischemia Pain, MOR: morphine, GAB: gabapentin, DRM: dermorphin, VE: vehicle.

Ligands: (admin route)	**Animal, assay, Cut-off latency, baseline latency, %MPE**	**%MPE or latency of Ligand (dose, post admin time)**	**%MPE or latency of Reference (dose, post admin time)**	**ED_{50} of Ligand**	**ED_{50} of Reference ligand**	**Ref.**
		Paw withdrawal latency assay (SNL rats)				
[Dmt[1]]DALDA	Male SD rats, R/H, 20s, 8.01 ±0.17s	15s (0.71 mg/kg, 120 min)	MOR: 12s (5.4 mg/kg, 30 min)	n/a	n/a	(297)
		Mechanical allodynia assay (SNL rats)				
Ligands: (admin route)	**Animal, assay, baseline threshold**	**Peak threshold of Ligand**	**Threshold of Reference**	**ED_{50} of Ligand**	**ED_{50} of Reference ligand**	**Ref.**
MGM-16 (s.c.)	Male ddY mice, no info	~1.4 g (0.4mg/kg, 30 min)	VE: ~0.75 g (30 min)	n/a	n/a	(300)
MGM-16 (p.o.)	Male ddY mice, no info	~1.3g (2.0mg/kg, 30 min)	GAB: ~0.4g (100mg/kg, 30 min) VE: ~0.65g (30 min)	n/a	n/a	(300)
[Dmt[1]]DALDA (s.c.)	Male Long Evans rats (250-450g), 0.25g - 15g, CPIP model	~15 g (0.5 mg/kg in 0.5 ml,180 min)	MOR: ~15 g (2 mg/kg, 40 min)	0.10±0.05 mg/kg	1.55±0.66 mg/kg	(343)
DeNo (i.t.)	Male SD rats (280-300 g), 40-150 g, ~ 60 g	~100 g (1nmol, 10 µl, 60 min)	DRM: ~130 g (1nmol, 10 µl, 45 min)	n/a	n/a	(319)
13a (i.t.)	Male CD1 mice (30-35 g), 0.6 g - 6 g, 6 g, CCI model	~6 g (0.05 nmol, 30 min)	MOR: ~4.5 g (35 nmol, 30 min)	0.003 nmol	7 nmol	(316)

1.2.6.3. Effects of non-bifunctional ligands on antinociceptive tolerance development

Apart from testing acute antinociception, some limited testing for antinociceptive tolerance was performed. Four different non-bifunctional analgesics were assessed in mice using repeated administration over 3 to 8 days using spinal antinociceptive assays (Table 12). In addition, tolerance to antinociceptive effects was measured as ED_{50} shifts compared to the first treatment day. Overall, these four non-bifunctional ligands showed reduced tolerance to morphine, which is similar to the results obtained with the bifunctional opioid analgesics (Table 12).

Table 12. The effects of non-bifunctional opioid analgesic ligands on sub-chronic administration and antinociceptive tolerance. Keys: ED_{50}: effective dose 50%, '~' shows an approximate value, other values are shown as Mean. TF: tail flick assay by radiant heat, TFL: tail-flick latency, s.c.: subcutaneous, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, i.t.: intrathecal, n/a: not applicable/available, MOR: morphine. (Note: Same experimental conditions/animals apply for the TF or WWTW assay with acute administration.

Ligand	Procedure summary	Result	%MPE of ligand	% MPE of morphine	Tolerance ED_{50} shift of Ligand	Tolerance ED_{50} shift of Morphine	Ref.
MMP-2200	MOR or MMP-2200 were administered at ED_{90} doses (b.i.d.) for 3 days and tested on day 4. Antinociceptive tolerance was measured using WWTW (55° C).	Less ED_{50} shifts than MOR (less tolerance than MOR)	Day 1: ~90%	Day 1: ~100%	4.8 fold	12.8 fold	(151)
AN81	AN81 (4 mg/kg i.v.) and MOR (4 mg/kg i.p.) once daily were administered for 5 days. TFL was measured days 1 and 5.	Morphine tolerance: Day 5 AN81 tolerance: Day 5	Day 1: ~100%, Day 5: 34.99%	Day 1: ~80% Day 5: ~0%	n/a	n/a	(184)
MGM-9	MGM-9 (1 mg/kg s.c.) and MOR (8mg/kg s.c.) were administered twice daily for 5 days. TFL was measured on days 1, 3 and 5.	Morphine tolerance: Day 3 MGM-9 tolerance: Day 5	Day 1: ~85% Day 3: ~70% Day 5: ~50%	Day 1: ~90% Day 3: ~55% Day 5: ~35%	n/a	n/a	(171)
BN-9	MOR (4 nmol i.c.v. and 2 nmol i.t.) and BN-9 (0.5, 1 and 2 nmol i.c.v. and i.t.) were administered once daily 8 days. TFL was measured daily.	Morphine tolerance: Day 4 BN-9 tolerance: No tolerance	Day 1: ~95% (i.c.v.), ~90% (i.t.) Day 4 to 8: no change	Day 1: ~90% (i.c.v.), ~95% (i.t.) Day 4:~50% (i.c.v.), ~80% (i.t.)	n/a	n/a	(178)

1.2.7. Opioid analgesics with an incomplete pharmacological profile

1.2.7.1. Pharmacological effects of opioid ligands with an incomplete profile

Apart from bifunctional and non-bifunctional opioid analgesics, some other OLs were reported over the described time-period, but only with incomplete pharmacological profiles. All of these sixteen OLs are described as MOP receptor agonists with some effects on other opioid receptors (Table 5). While these compounds were reported for their antinociceptive effects, no antinociceptive tolerance profiles have been reported for these ligands so far.

1.2.7.2. Antinociceptive effects of opioids with an incomplete pharmacological profile

Among the sixteen OLs with an incomplete pharmacological profile, ten compounds (62%) showed antinociceptive effects in three different animal species (Table 13). These compounds showed efficacy using intrathecal (5) and intravenous (3) administration, while none of these compounds was orally active. However, most of these ligands produced similar or better antinociception compared to morphine (Table 13). In addition to the tail-flick test, compounds 1 (323) and 11a (333) were also tested using the hot-plate assay where ligand 11a produced antinociception levels similar morphine. Ligand 1 reportedly produced full antinociception after intracerebroventricular (i.c.v.) administration, whereas it achieved only 50% antinociception after intravenous (i.v.) injection. In contrast, morphine produced more antinociception using i.v. administration compared to i.c.v. administration (Table 14). Therefore, it is important to note that opioid-induced antinociception can be significantly influenced by its route of administration.

Table 13. The antinociceptive effects using tail-flick assay of opioids with an incomplete pharmacological profile. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, other values are Mean, CL: cut-off latency, WWTW: warm water tail withdrawal assay, TF: tail flick assay by radiant heat, TFL: tail-flick latency, s.c.: subcutaneous, i.c.v.: intracerebroventricular, i.t.: intrathecal, i.v. intravenous. n/a: not applicable/available.

Ligands (admin route)	Animal, assay, Cut-off latency, baseline latency, %MPE	%MPE of Ligand (dose, post-admin time)	%MPE of Morphine (dose, post-admin time)	ED_{50} of Ligand	ED_{50} of Morphine	Ref.
10 (i.t.)	Male Whister rats (200-250 g), no info, % biphalin i.t., latency	99-100% (8.40 nmol, 30 min)	~40% (7.80 nmol, 20 min)	n/a	n/a	(321)
RV-Jim-C3 (i.t.)	Male ICR mice (20-25g), WWTW 52°C , 15 s, <7 s, %CL	~80% (10µg, 15 min)	n/a	3.92µg	n/a	(322)
1(i.c.v.)	Male CD-1 mice (25-30 g), TF, 15 s, no info, %CL	~85% (0.6 nmol, 30 min)	~25% (0.6 nmol, 30 min)	n/a	n/a	(323)
1(i.v.)	Male CD-1 mice (25-30 g), TF, 15 s, no info, %CL	51.3±7.3% (3 µmol, 30 min)	~90% (3 µmol, 30 min)	n/a	n/a	(323)
SR14150 (s.c.)	Male CD1 mice, TF, 15 s, 6.25 s, %CL	~75% (30 mg/kg, 60 min)	~95% (15 mg/kg, 60 min)	n/a	n/a	(344)
SR14150 (s.c.)	Male ICR SNL mice (20-25 g), 15 s, 4.17 s, latency	80% (10 mg/kg, 30 min)	93% (10 mg/kg, 30 min)	n/a	n/a	(296)
SR14150 (s.c.)	Male ICR mice (20-25g),TF, 15 s, 4.1 s, % CL	~100% (30 mg/kg, 60 min)	~90% (10 mg/kg, 30 min)	n/a	n/a	(283)

Ligands (admin route)	Animal, assay, Cut-off latency, baseline latency, %MPE	%MPE of Ligand (dose, post-admin time)	%MPE of Morphine (dose, post-admin time)	ED_{50} of Ligand	ED_{50} of Morphine	Ref.
SR16507 (s.c.)	Male ICR mice (20-25g),TF, 15 s, 4.11 s, % CL	~95% (3 mg/kg, 30 min)	~85% (10 mg/kg, 30 min)	n/a	n/a	(283)
Tapentadol (i.v.)	Beagle dogs, TF, 12 s, 3-5 s, %CL	~100% (6.81mg/kg, 10 min)	~100% (1.0mg/kg, 10 min)	4.3 mg/kg	0.71 mg/kg	(345)
13 (i.t.)	Male Wistar rats (300-350g), TF, 9 s, 1.4 s, latency	Max latency: ~8.5s (10nmol, 5µl, 30 min)	Max latency: ~7.5s (35nmol, 5µl, 30 min)	n/a	n/a	(330)
5 (AKG127)	ICR mice (15-20g), WWTW 52° C, 10 s, 3-6 s, %CL	68.4±14% (0.1µg, 5µl, 60 min)	n/a	n/a	n/a	(331)
26 (i.c.v.)	Male C57BL/6 mice, WWTW 55° C, 15 s, %CL	~90 % (30 nmol, 5 µl, 20 min)	~95% (10 nmol, 5 µl, 30 min)	1.98 nmol	2.35 nmol	(332)
22 (i.t.)	Male Wistar rats (250-300 g), TF, 9 s, 2 s, %CL	~95% (11 nmol, 30 min)	~85% (35 nmol, 30 min)	2.78 nmol	0.49 nmol	(293)
22 (i.v.)	Male C57BL6 mice (25-28 g), TF, 7s, 2s, % CL	~90% (10.8 µmol/kg, 30 min)	~30% (12.5µmol/kg, 30 min)	n/a	n/a	(293)

Table 14. Antinociceptive effects using hot-plate assay of opioids with an incomplete pharmacological profile. Keys: MPE: maximum possible effect, ED_{50}: effective dose 50%, '~' shows an approximate value, other values are Mean ± SEM (standard error mean), CL: cut-off latency, HP: hot plate, FEN: fentanyl, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, i.v.: intravenous.

Ligands (admin route)	**Animal, assay, Cut-off latency, baseline latency, %MPE**	**%MPE of Ligand (dose, post admin time)**	**%MPE of Morphine (dose, post admin time)**	**Ref.**
1 (i.c.v.)	Male CD-1 mice (25-30 g), 55.0 ± 0.1° C, 60s, no info, %CL	~100% (30 min)	~30% (30 min)	(323)
1 (i.v.)	Male CD-1 mice (25-30 g), 55.0 ± 0.1° C, 60s, no info, %CL	52.4±8.5% (30 min)	~80% (30 min)	(323)
11a (i.p.)	Albino Balb/c mice (25-30 g) (no info of sex), 55° C, 25 s, 7.8 s, %CL	~85% (40 mg/kg, 15 min)	~90% (10 mg/kg, 15 min)	(333)

1.3. Novel UTAS-derived opioids

Previously, the potential of combined bifunctional MOP receptor agonist / DOP receptor antagonist to reduce antinociceptive tolerance was highlighted (282,306,346). One of the most widely discussed ligands of this class is UFP-505 (H-Dmt-Tic-Gly-NH-Bzl) (306) (Fig. 2). It is believed that 'Dmt-Tic' peptides are required for DOP receptor antagonism and the spacer (Gly-NH) and third aromatic ring (Bzl) of UFP-505 is required for MOP receptor agonist activity (347,348). This compound was synthesised and characterised as compound 6 as part of a drug optimisation study to develop a DOP receptor antagonist and MOP receptor agonist (348). Later it was renamed to "University of Ferrara Peptide 505" (UPF-505) (349). In a recent study, Dietis and colleagues characterised that UFP-505 displays a variable expression-dependent efficacy (antagonist or partial agonist) at the DOP receptor (chapter 6) (350). Several previous studies reported the effects of opioids with mixed selectivity profile on multiple receptors based on the "Dmt-Tic" pharmacophore (348,351,352,353). After repeated intrathecal (i.t.) administration over a period of 3 days, UFP-505 produces less antinociceptive tolerance compared to morphine (354). On the other hand, UFP-505 increases antinociceptive tolerance and produces toxicity after intracerebroventricular (i.c.v.) administration (306). Moreover, UFP-505 was not an effective antinociceptive agent after systemic (subcutaneous or intravenous) administration (354). Novel analogues of UFP-505 with better bioavailability via clinically relevant routes of administration (e.g. subcutaneous, oral or intravenous) appear as attractive molecules for further development.

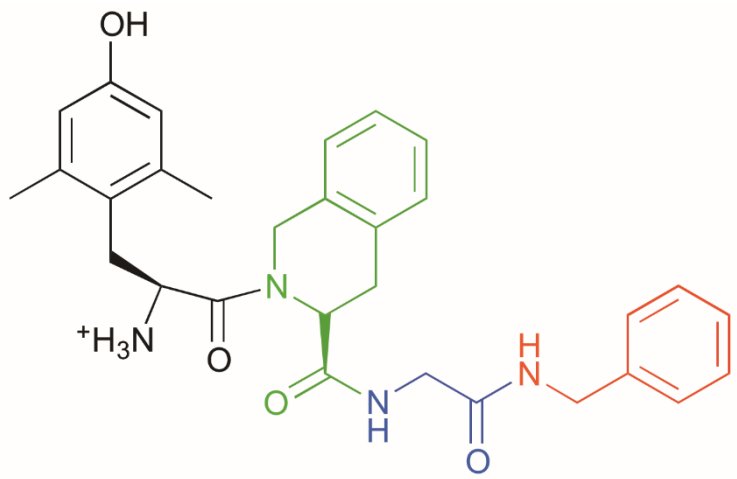


Figure 2. Chemical structure of UFP-505 (H-Dmt-Tic-Gly-NH-Bzl). Key: Dmt: 2',6'-dimethyl-l-tyrosine, Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, Gly: Glycine, Bzl: Benzyl.

So far, it was assumed that the Dmt-tic (benzyloxy-methylene group) pharmacophore of UFP-505 is essential for the agonistic effect on the MOP receptor and the antagonistic effect on the DOP receptor. To improve our understanding of the structure-activity relationship of UFP-505, six analogues of UFP-505 were synthesised by A/Prof Jason Smith (Division of Chemistry, University of Tasmania). These analogues included modifications of the "Tic" and hydrocarbon chain between the "NH" and "Bnz" groups, without any modification on the "Dmt" structure. Apart from providing a better understanding around the structure-activity relationship of this compound, these changes were intended to assess the suitability to alter the chemical structure of UFP-505 from a peptide to a peptidomimetic that might be effective via an oral route of administration.

1.4. Role of opioids in insulin homeostasis

In addition to the effects of morphine and other opioids on pain on antinociception, tolerance and behaviour, opioids are also used to mitigate pain in different metabolic disorders. However, the connection between the opioidergic system and the pancreatic metabolic system (e.g. glucose homeostasis) is important for diabetes patients. Diabetes mellitus is a metabolic disease

characterised by hyperglycemia, caused by either decreased pancreatic insulin secretion or an imbalance of insulin and glucose levels (355,356,357,358).

Globally, an ageing population is responsible for the highest opioid use, while this population is also increasingly susceptible to diabetes mellitus (359,360). Nearly one-third of diabetes patients experience neuropathic pain due to diabetes-induced nerve injury and nearly one-fifth of these patients suffer from chronic pain (361,362). Clinically used opioids like morphine or fentanyl are effective for the management of chronic or surgical pain, however, their effects in diabetic neuropathy are not fully understood (363). Opioid combinations (e.g. hydrocodone combinations, oxycodone, propoxyphene), topical preparations (e.g. lidocaine patches), anticonvulsants (e.g. gabapentin, pregabalin), antidepressants (e.g. amitriptyline, nortriptyline, venlafaxine, sertraline), ion-channel blockers, skeletal muscle relaxants (e.g. cyclobenzaprine) and different NSAIDs are also used to treat the patients with diabetic neuropathy (DPN) (364,365). Pregabalin, duloxetine and tapentadol are also known to be effective to manage neuropathic pain in patients experiencing DPN (366,367,368,369,370).

Different preclinical studies showed opioid-induced hypoglycaemia (reduction of blood glucose) in non-diabetic and diabetic animals (371,372,373,374,375), whereas several other animal studies showed opioid-induced hyperglycaemia (increase in blood glucose) (376,377,378,379,380). Studies show that MOP or KOP receptor agonist opioids induce hypoglycaemic effect (371,372,373,374,375) but DOP receptor agonist opioids can produce hyperglycaemia (380). In contrast, in healthy individuals as well as in non-diabetic patients opioids have only been reported to induce hypoglycaemia in the clinic (381,382,383,384,385,386). The opioid antagonist naloxone can help to prevent this hypoglycaemic response in healthy individuals as well as in diabetic patients (387,388). The

exact mechanism how opioids regulate glucose homeostasis is not clear but it might be related to a direct effect on insulin secretion as reported in a clinical case report (381) where unintentional exposure of methadone increased insulin secretion in an 11-month-old individual. Opioids therefore might have a short-term impact on the reduction of blood glucose levels in T2DM patients but the long-term effects of opioids on blood glucose levels are not clearly understood (389). Overall, opioids produce hypoglycaemic effects in either diabetic and non-diabetic patients or healthy people.

Activation of peripheral MOP receptors (by endogenous (e.g. β-endorphin) and exogenous opioids (e.g. loperamide) may increase glucose utilisation in the insulin-secreting pancreatic cells through the phospholipase C and protein kinase C (PLC-PKC) pathway (390). Similarly, activation of DOP or KOP receptor can reduce hyperglycaemia through increased glucose utilisation (372,391). Since insulin secretion can be blocked by an opioid antagonist like naloxone, it is likely that insulin secretion is affected by opioid receptors (392). Therefore, opioid receptors contribute to insulin-glucose homeostasis. However, while some studies showed stimulating effects of opioid agonists on insulin release from pancreatic cells (392,393,394,395,396,397,398), other studies reported opioid-dependent inhibition of insulin secretion (399,400,401,402,403,404). The discrepancy is likely a consequence of different methodologies or tools used in these studies, such as primary cell lines (e.g. β-cells) perfused organs, tissues or live animals. Therefore, understanding the specific roles of opioid receptors on insulin homeostasis is important to predict the effect of specific OLs on diabetic neuropathy. Therefore, the present study investigated the effects of different selective OLs on opioid-mediated insulin release in a pancreatic β-cell line.

1.5. Age-dependent antinociception and behavioural effects of opioids

Similar to diabetes and neuropathy, the prevalence of surgical and chronic pain is high among individuals over 60 years of age and therefore this population represent the largest cohort of opioid users (405). Since most preclinical studies are using adult animals to measure antinociceptive and behavioural effects of opioids, there is a consistent lack of available data from aged animals. This lack of data is particularly important for this high opioid-user age-cohort as it could inform the clinical use of opioids in older individuals. Morphine is a widely used opioid with different psychological and behavioural adverse effects but also with a very narrow therapeutic index (406,407,408). The pharmacokinetics of opioids significantly impact the balance between adequate pain-relief and minimal adverse effects (409,410,411). In the clinic, pharmacological pain management is approached by adjusting drug dose using different subjective calculations, but these are inconsistent and subjective to the specific clinical setting (i.e. hospital or clinic) (409,410,411,412,413). Some clinical studies observed differences in physiology, drug-receptor interactions and opioid kinetics between older and younger patients (414,415,416). Similar to those clinical studies, differential effects of morphine have also been described between older and younger animals, but these results are inconclusive (417,418,419,420,421,422). Most studies reported lower antinociception in older animals after administration of the same mg/kg doses of morphine (417,418,419,420,421), while one study showed no differences between aged and young animals (422). The reported variations are likely the consequence of experimental differences with regards to the use of animal species, age of animals, testing methods and other experimental conditions (417,418,419,420,421,422). In addition, in most preclinical studies, there is only sporadic evidence for simultaneous measurements of antinociception, behavioural effects and its connection to morphine pharmacokinetics. However, in the clinic, differential body composition and altered ADME parameters of elderly patients may result in different response to morphine compared to

younger patients. Drug-drug interactions are also prevalent among the elderly, as they are typically using multiple drugs simultaneously (423,424). In addition, age-dependent cytochrome P450 (CYP) enzyme activities and/or impaired renal function in older patients may contribute to opioid toxicity (424,425,426,427,428). Therefore, the evidence-based dosing of opioids in the elderly population is an urgent need to achieve effective pain-control while ensuring the safety of these patients.

CHAPTER TWO

Morphine dosing strategy plays a key role in the generation and duration of the produced antinociceptive tolerance

Preface to chapter two

Long-term use of morphine and other opioids in the clinic is limited due to its analgesic tolerance and other adverse effects, as described in chapter one. The role of morphine dosing in the manifestation of analgesic tolerance is not known over a period of long-term treatment, as most of the preclinical studies were conducted over a few days (*chapter one, Table 2*). The object of this study (*chapter two*) was to design an optimised dosing strategy (starting dose, follow-up dose after tolerance, the frequency of daily dosing and duration of treatment) to delay morphine-induced antinociceptive tolerance after chronic treatment.

2. Morphine dosing strategy plays a key role in the generation and duration of the produced antinociceptive tolerance

Abstract

Antinociceptive tolerance after repetitive administration of morphine severely limits its clinical use. Despite an increased mechanistic understanding of morphine tolerance, little is known about the influence of dosing regimens in its development. I hypothesised that the starting dose of morphine, dosing frequency and dose increments, influence antinociception and the manifestation of antinociceptive tolerance in rats. Male rats were randomly divided into four groups with different intermittent starting-doses of daily morphine (b.i.d.) followed by different increments of single-dose morphine upon development of antinociceptive tolerance, for 2-3 weeks: 2.5 (b.i.d.) → 5 → 10 → 15 mg/kg/day, 5 (b.i.d.) → 10 mg/kg/day, 5 (b.i.d.) → 15 mg/kg/day, 10 (b.i.d.) → 20 mg/kg/day. Antinociception was assessed daily pre-treatment and at several time-points over two hours post-administration, using tail-flick and hot-plate assays. Tolerance was defined as significant antinociceptive desensitisation and was presented as a significant reduction of the maximum and total antinociceptive efficacy upon morphine administration. Rats commenced on 2.5 mg/kg/day (b.i.d.) morphine developed tolerance faster than those started on 5 or 10 mg/kg/day (b.i.d.). Higher starting and maintenance doses of morphine compared to the previous dosing regimen produced prolonged antinociception and delayed tolerance. In contrast, lower starting and maintenance doses of morphine produced less total antinociception during the course of treatment and did not delay the onset of tolerance, but require smaller dose-increments to reach antinociception after the development of antinociceptive tolerance. These results suggest that morphine starting dose, dosing frequency, increments and timing determine the manifestation of antinociceptive tolerance and extent of antinociception. In addition, these results also highlight the need for generally standardised and validated assay protocols and procedures to compare different studies, as a prerequisite to translate pre-clinical results into the clinic.

Keywords: morphine; dosing regimen; antinociceptive tolerance; antinociception.

2.1. Introduction

Morphine is one of the most effective and widely prescribed drugs against chronic pain (22). The efficacy of long-term opioid treatment of chronic pain is debated in clinical practice due to the risk of unwanted side effects such as respiratory depression, sedation, chronic constipation, fluid retention, addiction, hyperalgesia, compromised immune function and antinociceptive tolerance (111,112). Tolerance manifests as decreased drug efficacy following repeated administration of the same drug (113). It necessitates increased dosing in order to maintain efficacy and represents the biggest problem for long-term pain management. To understand the factors influencing antinociceptive tolerance several distinct molecular mechanisms have been proposed, such as receptor trafficking and regulation, molecular desensitisation as well as receptor dimerisation (68,126,127,128,129,429). In addition, morphine can be administered in three different chemical forms, at different frequencies, using different routes of administration, all of which reportedly modify the extent of tolerance (140,154,180,183,429).

Nevertheless, there is very little information if and how morphine dosing itself can influence the development of its tolerance or its extent after long-term administration. One study that reported a clear link between morphine dosing (magnitude of starting dose and increment dose) and tolerance was (179), where Duttaroy and colleagues showed that a lower increment dose produced a higher shift in morphine's ED_{50} after 7 days of morphine's administration. They concluded that increment of the dose was a stronger predictor of tolerance than starting dose. In later studies the same group explored the effect of opioid efficacy in the development of tolerance and reported that high efficacy agonists produce less tolerance than lower efficacy agonists at equi-effective doses (430), whereas Madia and colleagues (431) and Dighe and associates (148) reported that the dosing method also plays a role in the development of

tolerance, with continuous administration (infusions/pellets) producing more tolerance than intermittent dosing. Although these reports insinuate the existence of an inverse relationship between dosing increment and opioid efficacy with tolerance development, the short-term administration protocols used in these studies do not reflect the molecular complexity of tolerance in long-term opioid exposure.

One major drawback of most pre-clinical studies that investigate the development of antinociceptive tolerance in rodents, is the methodology used for describing tolerance. One traditional method that has been widely used in the past is the cumulative administration of an opioid after pre-exposure to opioids and the description of a shift in the ED_{50} value of the generated dose-response curves. However, since the continuous exposure to opioids is known to be tightly related with molecular changes in opioid receptor behaviour, expression and desensitisation, it is highly likely that this traditional method of describing antinociceptive tolerance influences the outcome of the response that it is meant to describe. Although this method for describing antinociceptive tolerance might be acceptable in the area of basic pharmacology for the purpose of pharmacological profiling of an opioid, it does not reflect the way we understand and manage tolerance in a clinic. In a clinical setting, tolerance to opioids is detected as a reduction of the patient's analgesic response (efficacy) to non-analgesic levels after long-term administration of opioids (usually intermittent). The extent and grade of the patient's manifested tolerance is complete only after the patient returns to sufficient analgesia following the administration of a higher dose of the same opioid or the administration of a different opioid with higher efficacy. In an effort to bypass the above limitations in the exploration of tolerance, I designed a study that uses a method that reflects more accurately the way we understand and manage tolerance in the clinic, by using the significant drop of efficacy (response) and the overall analgesia experienced over a set period of time after repetitive

administration of opioids, as the two main markers of tolerance-development rather than the shift of the opioid's potency at a specific end-point. I believe that this method reflects more accurately the method we use today in the clinic to detect and manage opioid tolerance, and therefore the conclusions from this study might help us understand better the existing relationship between opioid dosing regimen and opioid tolerance.

2.2. Methods

2.2.1. Animal maintenance and care

Twenty-four male Sprague-Dawley (SD) rats (238.0 ± 6.2 g, 8 weeks), supplied by the animal services of the University of Tasmania were housed as three littermates per cage at 22 ºC with 50-60 % humidity under an automated 12 hour day/night cycle (lights on at 7:00 am) with free access to food (Barastoc rodent cubes, Ridley Corporation, Melbourne, Australia) and water. Only male rats were used to avoid any possible effects of the oestrous cycle of female rats (336). Measurement of nociception was performed according to the ethical guidelines for investigations of experimental pain in conscious animals (432,433). Moreover, all procedures and handling were approved by the University of Tasmania Animal Ethics Committee (A0013864) and were conducted according to *The Australian Code for the Care and Use of Animals for Scientific Purposes* (434). The experiments were also in compliance with the *ARRIVE* guidelines (435).

2.2.2. Treatment protocol

Weights were recorded daily immediately prior to experiments in order to determine the dosage for each rat and it was calculated based on their body weights from a commercially available 30 mg/ml morphine sulphate solution (Hameln Pharmaceuticals GmbH, Germany). The drug was administered by daily subcutaneous injections between the space between left thigh and the spine. All rats were randomly divided into four subgroups and different sub-groups received different dosing-regimes of morphine (Fig. 3).

Group 1 (n = 5): Morphine sulphate 2.5 mg/kg/day (twice daily, b.i.d.) for 5 days, followed by a single dose of 5 mg/kg/day from day 6 to 10, 10 mg/kg/day from day 11 to 18, and 15 mg/kg/day from day 19 to 23.

Group 2 (n = 5): Morphine sulphate 5 mg/kg/day (b.i.d.) for 5 days, followed by a single dose of 10 mg/kg/day from day 6 to 14.

Group 3 (n = 8): Morphine sulphate 5 mg/kg/day (b.i.d.) for 5 days, followed by a single dose of 15 mg/kg/day from day 6 to 14.

Group 4 (n = 6): Morphine sulphate 10 mg/kg/day (b.i.d.) for 10 days, followed by a single dose of 20 mg/kg/day from day 11 to 14.

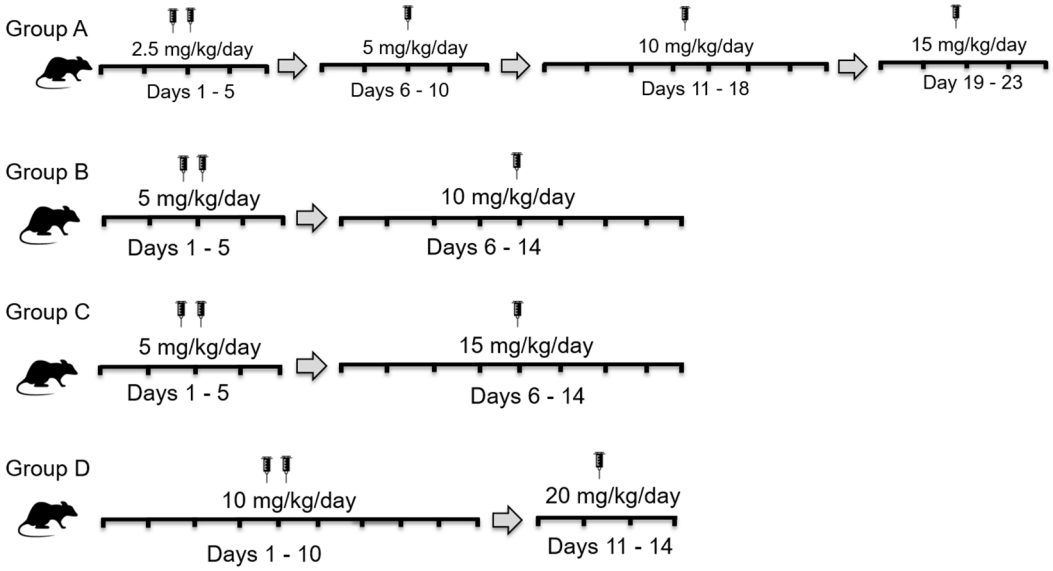


Figure 3. Schematic diagram of morphine dosing strategy. Twenty-four Sprague Dawley rats were randomly divided into four groups (A, B, C and D). All of these animals were treated with morphine at multiple escalating doses: 2.5 (b.i.d.) → 5 → 10 → 15 mg/kg/day (group A), 5 (b.i.d.) → 10 mg/kg/day (group B), 5 (b.i.d.) → 15 mg/kg/day (group C) and 10 (b.i.d.) → 20 mg/kg/day (group D) over 14 to 23 days, as described in Methods.

The intensity of laboratory illumination was reduced prior and during experiments to minimise any potential discomfort to the animals. At the end of the observation period, animals were anesthetised with 5% (w/v) isoflurane in oxygen at a flow rate of 1 L/min, until the animal was unconscious (usually 5-7 min), before decapitated.

2.2.3. Assessment of antinociception

Nociceptive thresholds were determined by two independent assays performed (tail-flick & hot plate) using commercially available tail-flick and hot/cold plate assay equipment (Ugo Basile, Comerio, Italy). The maximum exposure to the nociceptive thermal stimulus was 15 sec for the tail-flick (basal latency: 3.93 ±0.18 sec) and 30 sec for the hot-plate assay (basal latency: 5.69 ±0.15 sec). The infrared intensity of the tail-flick photocell was set at 30, whereas the plate temperature of the hot-plate was set at 54 ±0.5 °C. Every rat was tested immediately prior to morphine administration to obtain the basal measurement and at 15, 30, 60 and 120 min post-administration in both assays on the first and the last treatment day for every morphine dose. On all other days, the rats were tested pre- (basal) and 30 min post-administration of morphine. Nociception measurements were conducted in a blinded manner and the mean of three independent measurements for each time-point with a 1 min interval between measurements was recorded to minimise the 'handling' effects. The maximum possible effect (MPE) was defined as MPE % or antinociception = 100 X [(test latency – baseline latency)/(cut-off time – baseline latency)] as previously described (436). Where "baseline latency" is the basal measurements prior to injections of that particular test-day or test-time. Basal latencies (in seconds) from day 1 to day 14 of all group of rats were not significantly different from day 1 (Fig.4). Typically, antinociceptive tolerance is described either as the percentage of the maximum possible antinociceptive effect (MPE %), area under the curve (AUC) of the antinociceptive effect, a shift of its dose-response curve to the right, or the fold-shift of its median effective dose (ED_{50}) from naive to tolerant animals (140,149,429). Here, the antinociceptive effect was measured by two different methods (MPE % and AUC) in order to express changes in time-dependent and total efficacy over the course of treatment.

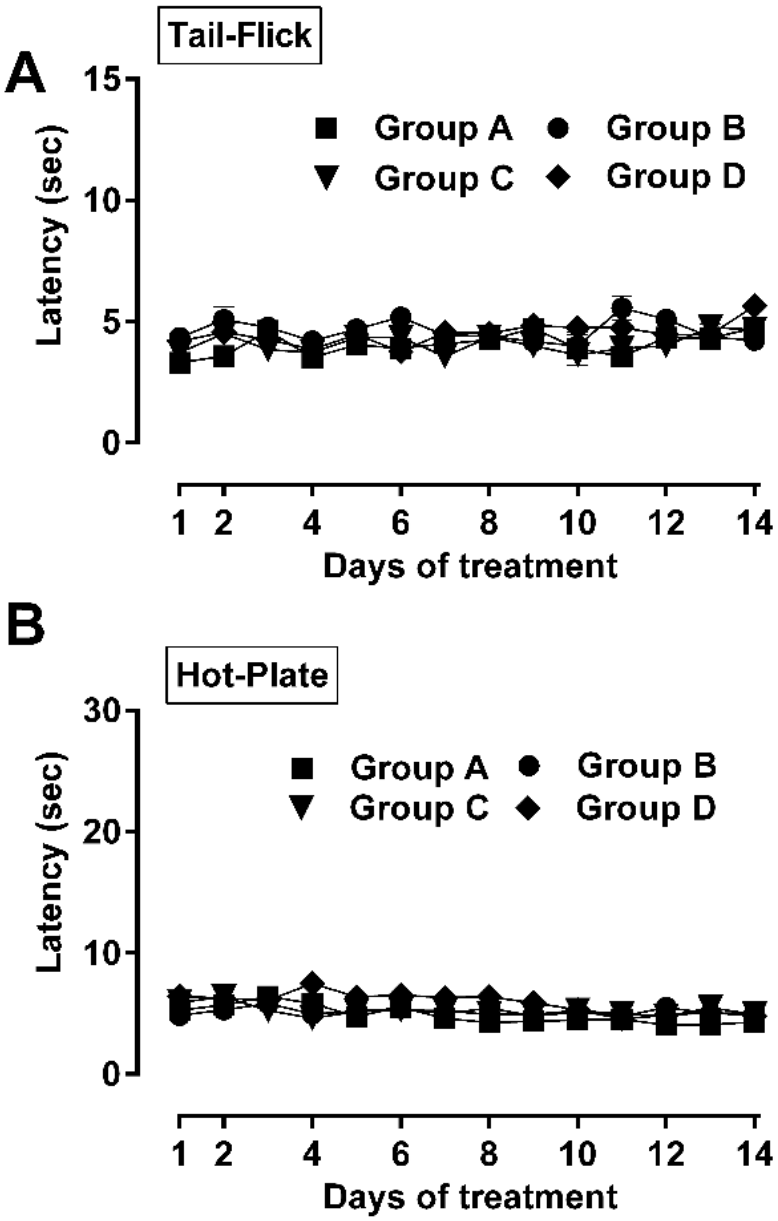


Figure 4. Basal antinociception of rats. Antinociceptive latencies of morphine in male Sprague Dawley rats at pre-administration (basal) were measured using the tail-flick (A) and hot-plate (B) assays. The antinociception is represented as latency in seconds (sec). Group A, B, C and D represent different morphine-treated rats as described in *Methods*. All data are represented as Mean ± SEM derived from n = 5 - 8 rats. No statistically significant differences were observed against day 1 using one-way ANOVA with Dunnett's multiple comparisons tests.

2.2.4. Statistical analysis

Data are expressed as mean ± SEM and analysed by one-way ANOVA with Dunnett's or Tukey's multiple comparisons (post hoc) test or unpaired t-test with Welch's correction, using GraphPad Prism V6 software (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons tests were employed when F was achieved $p < 0.05$ and there was no significant variance in homogeneity. A '*p'* value less than 0.05 was considered statistically significant.

2.3. Results

2.3.1. Morphine-induced tolerance is delayed by higher morphine doses

To gain a basic understanding of morphine-induced tolerance in my experimental setting, rats were treated with two daily morphine doses of up to 10 mg/kg/day and the resulting antinociception was assessed daily over a period of 5 days. Before morphine administration, no significant differences in basal antinociception (day 0) were observed in the three dosing groups (Fig. 5 A-C), in both the tail-flick (circles) and hot-plate assays (squares). Upon morphine administration on day 1, all animals produced a significant antinociceptive response within 30 min of injection. However, only animals treated with 5 mg/kg/day (b.i.d.) (Fig. 5 B) or 10 mg/kg/day (b.i.d.) (Fig. 5 C) reached 100 % antinociception, while in the dose-group of 2.5 mg/kg/day (b.i.d.), morphine produced only 43.6 ± 7.4 % and 30.6 ± 2.4 % antinociception in the tail flick and hot plate assays respectively on day 1 (Fig. 5 A).

Overall, there was a trend that antinociception in the tail-flick assay measured higher antinociception levels compared to the hot plate assay. These differences were significant for the 5 mg/kg/day dose group on days 1, 2, 3 and 5 (e.g. on day 5: unpaired t-test; $p < 0.05$; $t(9.65) = 2.62$; Fig. 5 B) and in the 10 mg/kg/day dose group on day 3 (unpaired t-test; $p < 0.05$; $t(5.00) = 2.74$; Fig. 5 C), while for the 2.5 mg/kg/day dose group no significant differences were observed (Fig. 5 A). The development of antinociceptive tolerance in this system was defined as a significant reduction of antinociception compared to the response on day 1. For the lowest morphine dose, significant tolerance already manifested from day 2 and day 3 onwards in the tail flick and hot plate assays respectively (Fig. 5 A).

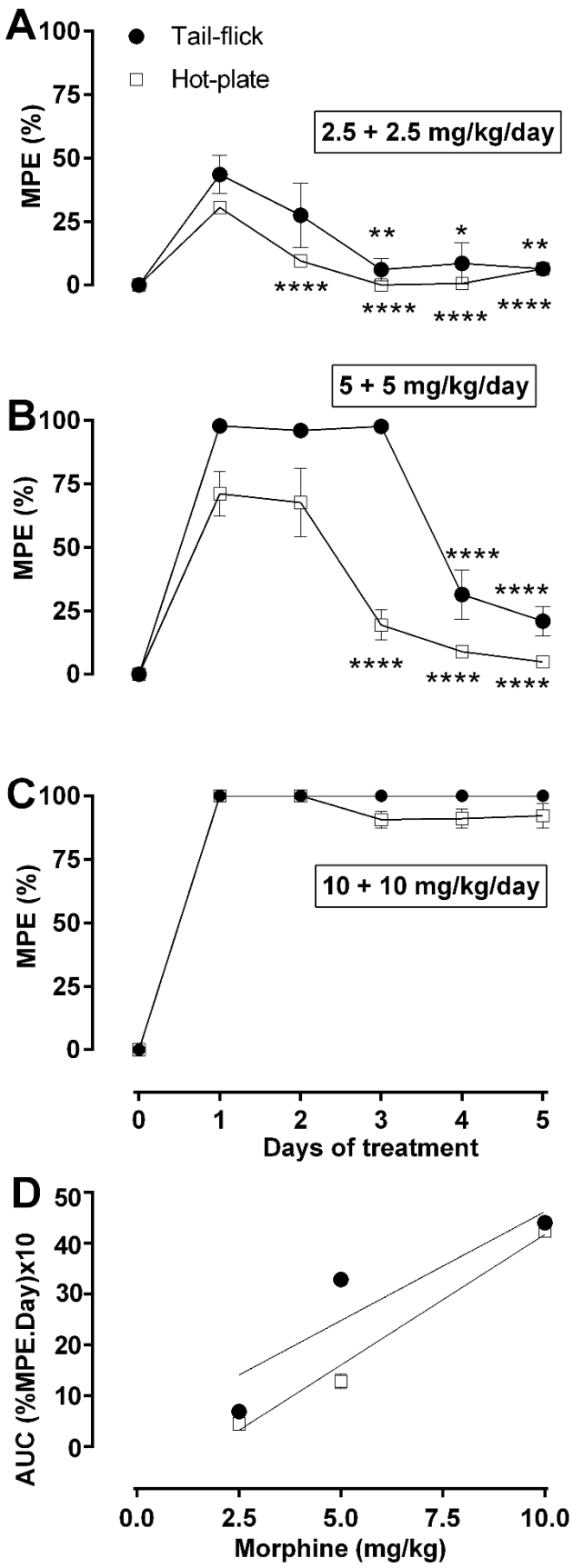


Figure 5. Morphine-induced tolerance. Antinociceptive effects of twice daily (8 am, 5 pm) s.c. injections of morphine sulphate were tested in tail-flick (TF, circles) and hot-plate (HP, squares) assays in male Sprague Dawley rats. Antinociception was calculated as maximum possible effect, % MPE, as described in Methods. Antinociception was measured every morning (8-10 am) over 5 days, 30 min post-administration of different morphine doses: 2.5 mg/kg/day b.i.d. (A), 5 mg/kg/day b.i.d. (B), and 10 mg/kg/day b.i.d. (C). The areas under the curves (AUC) from A-C were plotted against the corresponding morphine doses for each assay used (D), presenting the dose-dependent increase of total antinociception from 5 treatment days. All data are represented as mean ±SEM derived from n = 5 (A), n= 13 (B), n= 6 (D) rats per group. Statistically significant differences compared to day 1 were calculated using one-way ANOVA with Dunnett's multiple comparisons test (*p < 0.05, **p < 0.01 and ****p < 0.0001). Error bars are present in all graphs but are sometimes too small to be visible.

In the 5 mg/kg/day (b.i.d.) morphine group tolerance was delayed by one day from day 3 in the tail flick and day 4 in the hot plate assay (Fig. 5 B). In contrast, 10 mg/kg/day (b.i.d.) morphine produced full antinociception (100 %) over the entire 5-day period with no significant tolerance observed (Fig. 5 C). To correlate morphine dose with antinociception, the areas under the curves (AUC) of Fig. 5 A-C were plotted against the respective morphine doses and good correlations between antinociception and morphine dose were observed for both the hot plate ($R2 = 0.984$) and tail flick assay ($R2 = 0.821$) (Fig. 5 D).

2.3.2. Higher starting-doses and larger dosing-increments delay antinociceptive tolerance and produce higher total antinociception levels

To further investigate the effect of morphine-dosing on the development of tolerance during the whole treatment period of 14 days, increasing concentrations of morphine were tested sequentially over a period of 2 weeks, while antinociception was measured by the tail flick assay. As described before (Fig. 5 A), 2.5 mg/kg/day (b.i.d.) daily morphine doses produced tolerance from day 3 onwards (Fig. 5 A). Under these conditions, a single morphine dose of 5 mg/kg/day at day 6 restored antinociception to previous maximum levels (Fig. 6 A) as shown by the lack of significant difference in the levels of antinociception between days 1 and 6. With this reduced injection-frequency of the same daily morphine dose, tolerance reappeared immediately from day 7 and this effect could be again reverted by increasing the morphine dose to a single 10 mg/kg/day injection. Comparable to the previous lower doses, tolerance immediately reappeared one day later on day 12 (Fig. 6 A).

The same paradigm was followed in the higher starting-dose group of 5 mg/kg/day (b.i.d.) morphine, which produced 100 % antinociception on day 1 (Fig. 6 B). Consistent with our previous results (Fig. 5 B), tolerance developed at day 4 and was overcome by a single

morphine dose of 10 mg/kg/day from day 6. Using this reduced frequency of administration by keeping the same daily dose, tolerance developed from day 9 until the end of the observation period (Fig. 6 B). Interestingly, under these conditions, antinociception (latencies in seconds) on day 14 was still significantly higher than the basal antinociception on day 0 (unpaired t-test; $p < 0.05$; $t(3.57) = 3.20$).

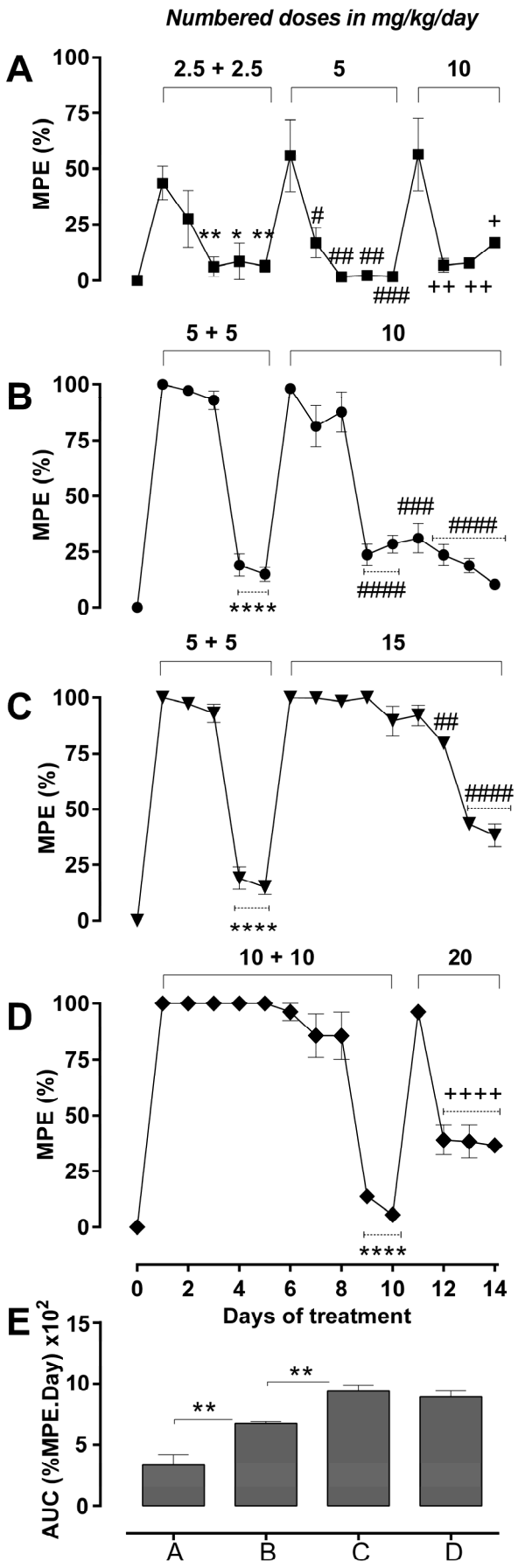


Figure 6. Dose-dependent morphine tolerance using tail flick assay. Antinociceptive effects of morphine were measured in male Sprague Dawley rats 30 min post-administration (s.c.) using the tail-flick assay. Antinociception was calculated as percentage of the maximum possible effect. Morphine was administered at multiple escalating doses: 2.5 (b.i.d.) → 5 → 10 mg/kg/day (A), 5 (b.i.d.) → 10 mg/kg/day (B), 5 (b.i.d.) → 15 mg/kg/day (C) and 10 (b.i.d.) → 20 mg/kg/day (D) over 14 days, as described in Methods. (E) The areas under the curves (AUC) of the antinociceptive effects of (A)-(D) were compared. All data are represented as Mean ± SEM derived from n = 5-6 rats per group. Statistically significant differences compared against day 1 (*p < 0.05, **p < 0.01 and ****p < 0.0001), against day 6 (#p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001) or against day 11 (+p < 0.05, ++p < 0.01 and ++++p < 0.0001) are shown for the respective groups, as produced from one-way ANOVA with Dunnett's multiple comparisons tests. Additionally, significant differences between AUCs of different groups (A, B, C and D) are shown as **p < 0.01 and were calculated using one-way ANOVA with Tukey's multiple comparisons test. Error bars are present in all graphs but are sometimes too small to be visible.

Since increasing the morphine dose after the first occurrence of tolerance appeared to produce increased levels of antinociception, I increased the second morphine dose of the next group even further, to 15 mg/kg/day (Fig. 6 C) from day 6, in order to evaluate whether a higher increment would produce higher antinociception. As seen before (Fig. 6 B), tolerance only occurred from day 6 in this treatment regime, manifesting a delayed tolerance and a higher total antinociception. Nevertheless, when 15 mg/kg/day morphine was administered from day 6 onwards, the animals only became tolerant at day 12 (Fig. 6 C) and antinociception (latencies in seconds) on day 14 was still significantly higher than the basal response of day 0 (unpaired t-test; $p < 0.05$; $t(2.30) = 7.18$) (Fig. 6 C). In this paradigm, antinociception on day 14 (38.4 ± 5.2%) was not significantly different from the antinociception on day 1 (43.6 ± 7.4 %) of the lowest starting-dose group (2.5 mg/kg/day b.i.d.).

For the last group, a starting dose of morphine 10 mg/kg/day (b.i.d.) produced significant antinociception for 8 days, until significant tolerance developed on day 9. At day 10, antinociception was not statistically different from the basal value on day 0 (Fig. 6 D). Following the manifestation of tolerance, a single morphine dose of 20 mg/kg/day on day 11 was able to produce a maximum antinociception (100 %) (Fig. 6 D). However, a subsequent morphine dose was unable to sustain antinociception and tolerance quickly developed at day 12, which was sustained until the end of the test. Nevertheless, the antinociception level (latencies in seconds) at day 14 was still significantly higher than the basal at day 0 (unpaired t-test; $p < 0.05$; $t(2.87) = 4.00$) (Fig. 6 D), which demonstrates that a 14-day high-dose morphine treatment can manifest not only higher total antinociception but also delayed tolerance compared to low-dose treatments.

The total morphine-induced antinociception of the entire 2-week period from all four different dosing groups was calculated as AUC values and compared (Fig. 6 E), with significant differences observed between groups (Fig. 6; Group A vs. Group B, Group B vs. Group C, or Group A vs. Group C; One way ANOVA with Tukey's multiple comparisons test; $p < 0.01$; $F(3, 15) = 24.64$). The corresponding latencies (in seconds) of Fig. 6 are shown in Fig. 7.

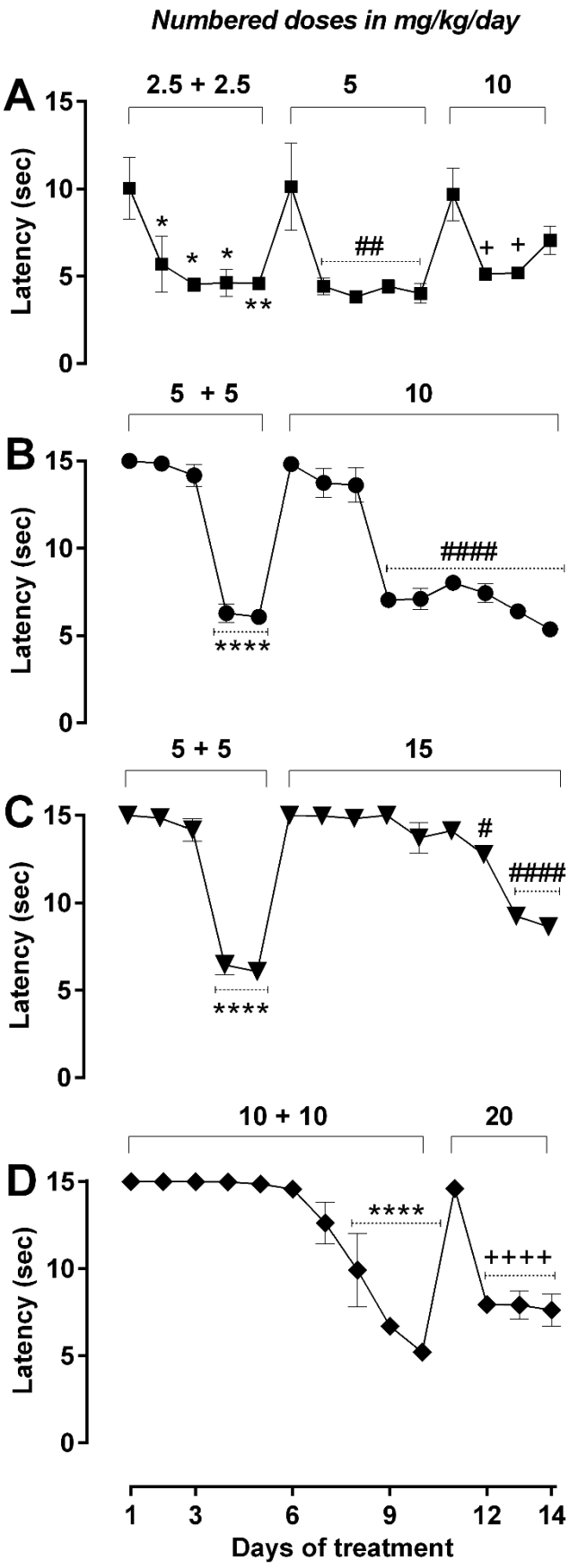


Figure 7. Dose-dependent morphine tolerance using tail-flick assay. Antinociceptive latencies of morphine in male Sprague Dawley rats 30 min post-administration (s.c.) were measured using the tail-flick assay. The antinociception is represented as latency in seconds (sec). Morphine was administered at multiple escalating doses: 2.5 (b.i.d.) $\rightarrow$ 5 $\rightarrow$ 10 mg/kg/day (A), 5 (b.i.d.) $\rightarrow$ 10 mg/kg/day (B), 5 (b.i.d.) $\rightarrow$ 15 mg/kg/day (C) and 10 (b.i.d.) $\rightarrow$ 20 mg/kg/day (D) over 14 days, as described in Methods. All data represent Mean ± SEM derived from n = 5-6 rats per group. Statistically significant differences compared against day 1 are shown as *p < 0.05, **p < 0.01 and ****p < 0.0001 or against day 6 as #p < 0.05, ##p < 0.01 and ####p < 0.0001 or against day 11 as +p < 0.05 and ++++p < 0.0001 using one-way ANOVA with Dunnett's multiple comparisons test. Error bars are present in all graphs but are sometimes too small to be visible.

In contrast to the tail flick assay, which is largely a measure of spinal-mediated pain perception (135), the hot plate assay is reflective of supra-spinal-mediated pain perception (136). To see if the observed dose-dependency of tolerance initiation could also be replicated using the hot plate assay, morphine-induced antinociception was measured in the 4 different dosing paradigms utilised in the previous experiment (Fig. 8 A-D). Comparable to the results from the tail flick assay, 2.5 mg/kg/day (b.i.d.) morphine induced a short period of antinociception on day 1 (30.6 ± 2.4 %), while tolerance was observed from day 2 to day 5 (Fig. 8 A). A subsequently reduced administration frequency of the same daily morphine dose (5 mg/kg/day) from day 6 was unable to overcome the induced tolerance. Only when the morphine dose was doubled to 10 mg/kg/day on day 11, was limited antinociception induced (21.9 ± 5.8 %). However, tolerance reappeared from day 13 (Fig. 8 A).

At the higher starting dose of 5 mg/kg/day morphine (b.i.d.), tolerance developed from day 3 and was reversed on day 6 by decreasing the administration frequency of the same daily dose to 10 mg/kg/day, which produced similar antinociception levels ($p = 0.696$) compared to day 1 (Fig. 8 B). Using this stage, tolerance developed after 2 days and persisted for the residual observation period. However, under these conditions, antinociception on day 14 (latencies in seconds) was still significantly higher than basal antinociception on day 0 (unpaired t-test; $p < 0.05$; $t(2.52) = 4.46$) (Fig. 8 B). Analogous to the tail flick experiment (Fig. 8 C) the second dose in the hot plate experiments was increased to a single dose of 15 mg/kg/day (Fig. 8 C). In contrast to the tail flick experiments, this increase did not delay the re-appearance of tolerance compared to the lower dose of 10 mg/kg/day (Fig. 8 C). Instead, the development of tolerance was remarkably similar to between the 10 and 15 mg/kg/day dose regimes (Fig. 8 B, C). Similar to the lower morphine dose (Fig. 8 B), 15 mg/kg/day on day 14 produced a trend towards slightly increased antinociception (14.6 ± 5.9 %) compared to day 0, which however did not

reach statistical significance.

When the dosing regimen was increased to a starting dose of 10 mg/kg/day morphine (b.i.d.), significant levels of antinociception were observed until day 5. While tolerance increased gradually from day 6 to day 10, antinociception on days 9 and 10 was statistically not different to the basal antinociception on day 0 (Fig. 8 D). With the reduction of the administration-frequency of the same daily morphine dose to 20 mg/kg/day from day 11 onwards, a significantly lower level of antinociception (78.1 ± 7.7 %; unpaired t-test; $p < 0.05$; $t (2.85) = 4.00$) compared to day 1 was only observed on day 11 before tolerance was evident from day 12 onwards (Fig. 8 D). However, under these conditions, antinociception on day 14 (latencies in seconds) remained significantly higher compared to basal antinociception on day 0 (unpaired t-test; $p < 0.001$; $t (4.93) = 22.66$).

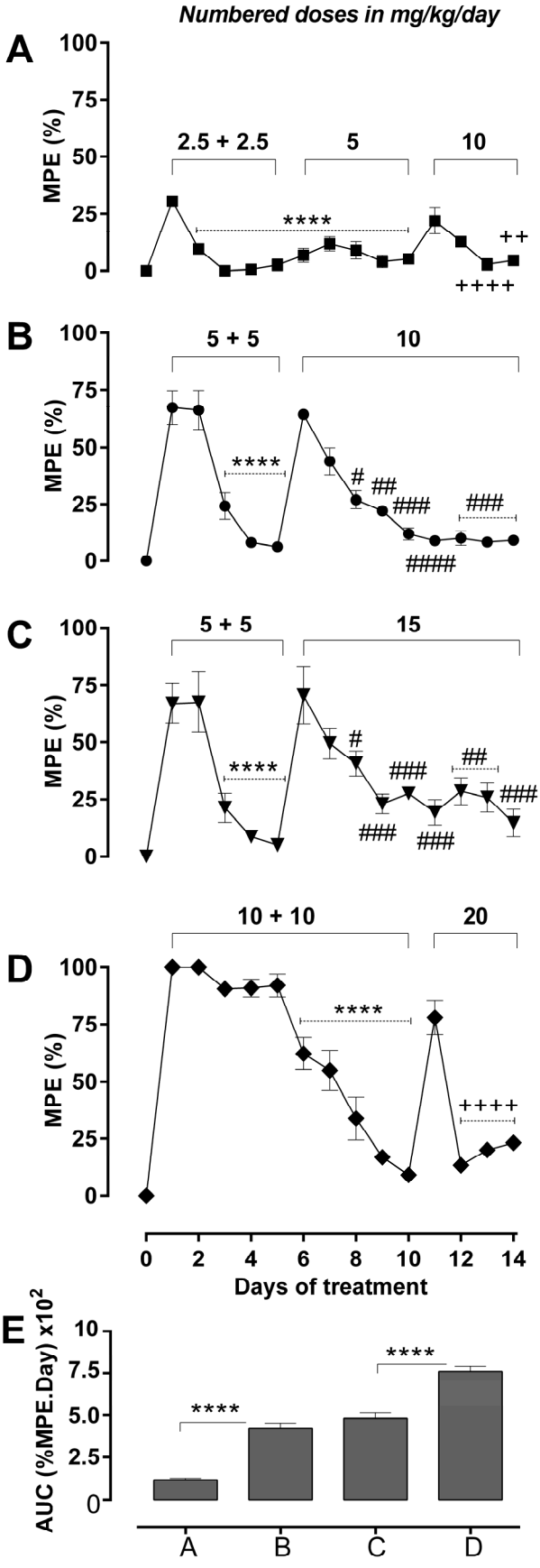


Figure 8. Dose-dependent morphine tolerance using hot-plate assay. Antinociceptive effects of morphine were measured in male Sprague Dawley rats 30 min post-administration (s.c.) using the hot plate assay. Antinociception was calculated as percentage of the maximum possible effect. Morphine was administered at multiple escalating doses: 2.5 (b.i.d.) $\rightarrow$ 5 $\rightarrow$ 10 mg/kg/day (A), 5 (b.i.d.) $\rightarrow$ 10 mg/kg/day (B), 5 (b.i.d.) $\rightarrow$ 15 mg/kg/day (C) and 10 (b.i.d.) $\rightarrow$ 20 mg/kg/day (D) over 14 days, as described in Methods. (E) The areas under the curves (AUC) of the antinociceptive effects of (A)-(D) were compared. All data are represented as Mean ± SEM derived from n = 5-8 rats per group. Statistically significant differences compared against day 1 ($^{****}p < 0.0001$), against day 6 ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$) or against day 11 ($^{++}p < 0.01$ and $^{++++}p < 0.0001$) are shown for the respective groups, as produced from one-way ANOVA with Dunnett's multiple comparisons test. Additionally, significant differences between AUCs of different groups (A, B, C and D) are shown as $^{****}p < 0.0001$ and were calculated using one way ANOVA with Tukey's multiple comparisons test. Error bars are present in all graphs but are sometimes too small to be visible.

When the four dosing groups were compared regarding the total morphine-induced antinociception for each animal over the entire observation period, significant differences in antinociception were observed between the first and the second dosing group (Fig. 8 E; A versus B; using one way ANOVA with Tukey's multiple comparisons test; $p < 0.0001$; $F (3, 13) = 108.3$), as well as the third and fourth group (Fig. 8 E; C versus D, using one way ANOVA with Tukey's multiple comparisons test; $p < 0.0001$; $F (3, 13) = 108.3$). No statistical difference was detected between the AUCs of the second and third group (Fig. 6 E; B versus C). The corresponding latencies (in seconds) of Fig. 8 are presented in Fig. 9.

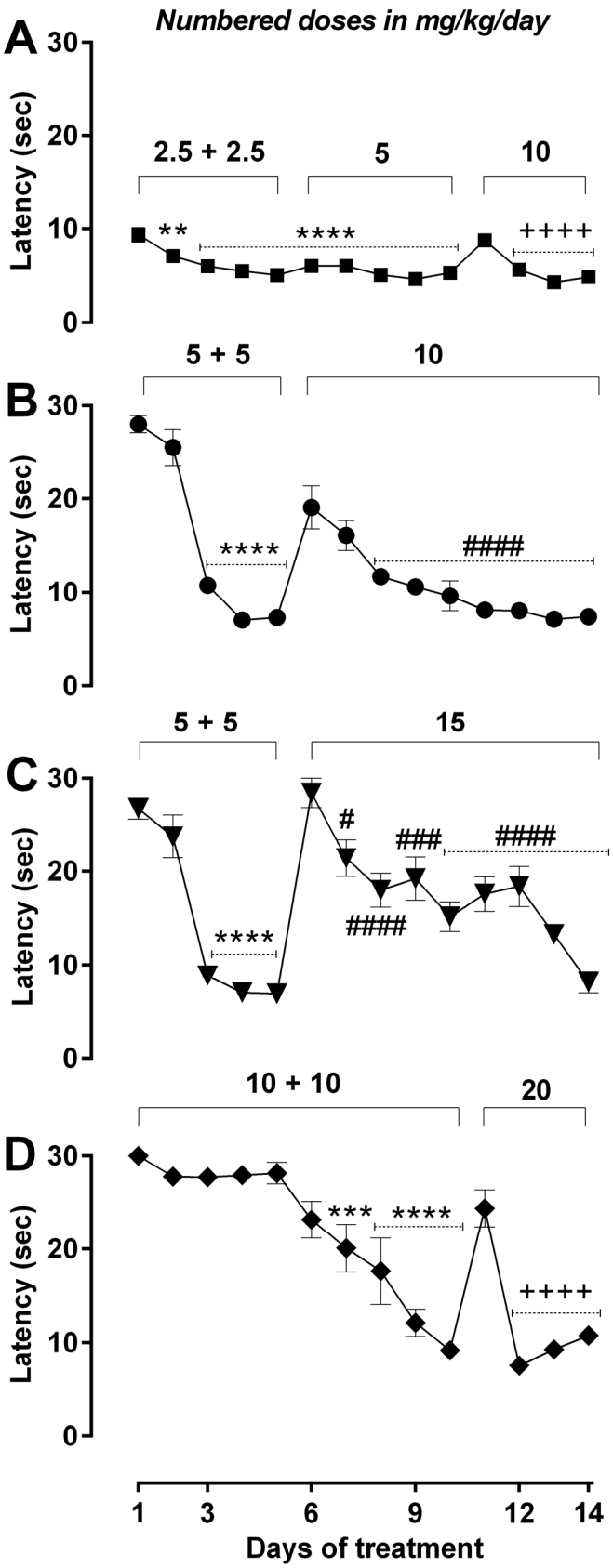


Figure 9. Dose-dependent morphine tolerance using hot-plate assay. Antinociceptive latencies of morphine in male Sprague Dawley rats 30 min post-administration (s.c.) were measured using the hot-plate assay. The antinociception is represented as latency in seconds (sec). Morphine was administered at multiple escalating doses: 2.5 (b.i.d.) $\rightarrow$ 5 $\rightarrow$ 10 mg/kg/day (A), 5 (b.i.d.) $\rightarrow$ 10 mg/kg/day (B), 5 (b.i.d.) $\rightarrow$ 15 mg/kg/day (C) and 10 (b.i.d.) $\rightarrow$ 20 mg/kg/day (D) over 14 days, as described in *Methods*. All data represent Mean ± SEM derived from n = 5-8 rats per group. Statistically significant differences compared against day 1 are shown as $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ or against day 6 as $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ or against day 11 as $^{++++}p < 0.0001$ using one-way ANOVA with Dunnett's multiple comparisons test. Error bars are present in all graphs but are sometimes too small to be visible.

2.3.3. Time-resolved antinociception by a single morphine dose in tolerant and non-tolerant animals

The observed dosing-dependent manifestation of antinociceptive tolerance throughout the treatment period of two weeks (Fig. 5, 6 and 8), was calculated from measurements of nociception taken at 30 min post-administration of morphine since the maximum efficacy of antinociception was observed during this time mark. This does not exclude the possibility that compensatory mechanisms developed during the treatment period could have altered morphine's pharmacokinetics and opioid receptor regulation, which might have led to antinociception and tolerance at different time-points post-injection. In order to examine this possibility, a time-resolved analysis of antinociception response to each morphine injection over a 2-hour post-administration period was used to compare the first day of treatment (non-tolerant) to the last day of the tolerant phase (day 5 for the 2.5 and 5 mg/kg/day groups; day 10 for the 10 mg/kg/day group). My results indicate that maximal antinociception was achieved at 30 min post-morphine administration on all days tested throughout the 2-week test period (Fig. 10), which therefore represents a precise and appropriate time-point to measure maximal antinociception after a single subcutaneous injection. This time-resolved analysis on the non-tolerant vs the tolerant treatment day in each group revealed that all three morphine starting-doses (2.5, 5 and 10 mg/kg/day b.i.d.) induced a dose-dependent antinociception in the pre-tolerance phase (Fig. 10). Consistent with the previous results, tolerance was evident on day 5 for the first two dose-groups, although the total antinociception of day 5 at the 5 mg/kg/day dose group was significantly higher than that of 2.5 mg/kg/day. Interestingly, although tolerance was developed 5 days later in the 10 mg/kg/day group (day 10), the total antinociception produced from a single-dose at day 10 was similar to the other groups. Rats showed no residual antinociception after 24 h of morphine administration as evidenced by the lack of difference between their basal antinociception levels at day 1 and day 5 or 10 (Fig. 10

A-C, E-G). The significant differences detected in antinociception were also evident by the comparison of the AUC of total antinociception among groups in both assays (Fig. 10 D, H).

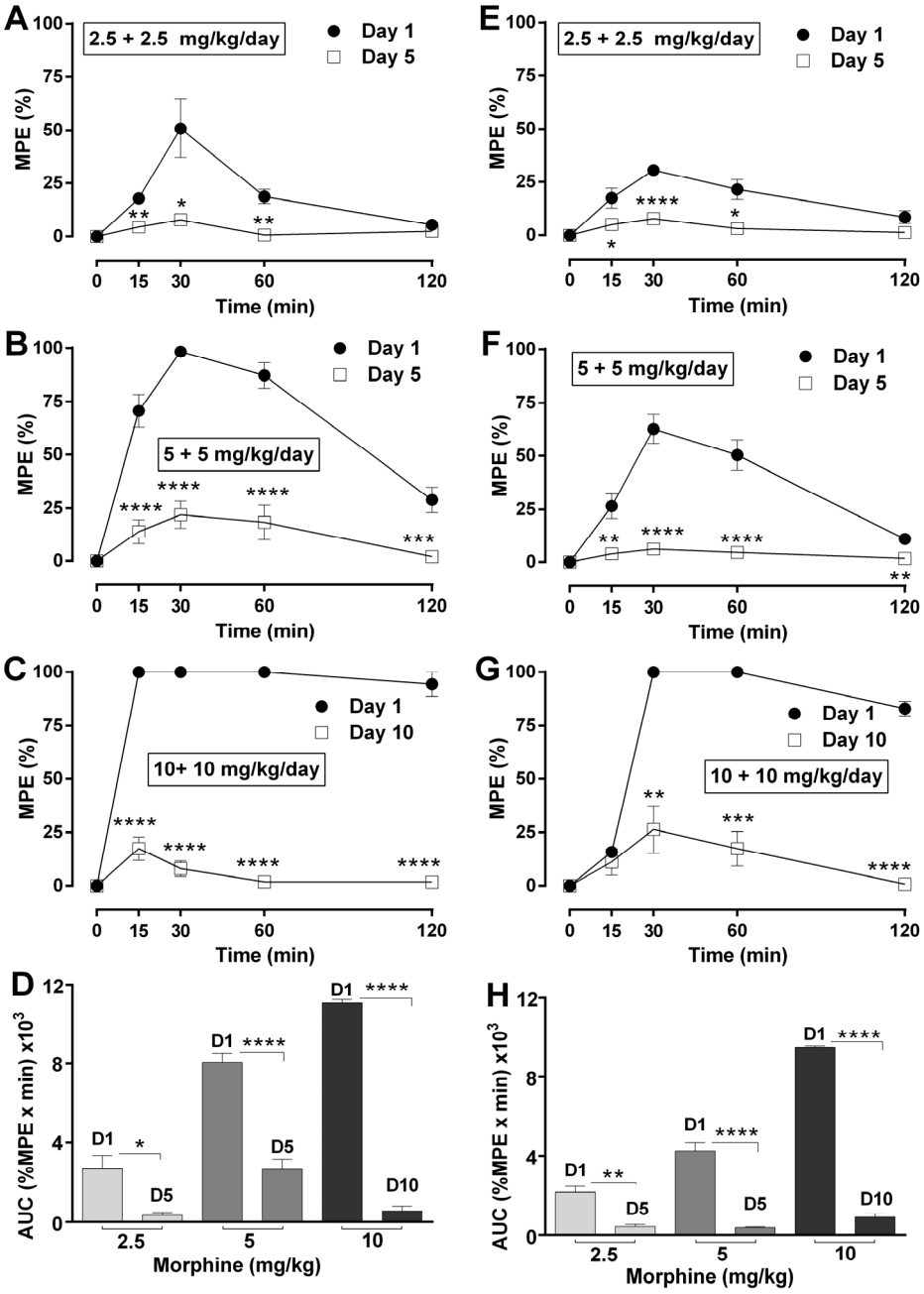


Figure 10. Time-resolved morphine tolerance. Antinociceptive measurements of a single-dose morphine from the first treatment day (day 1) and last treatment day (day 5 or 10) of each dosing group in both assays used: 2.5 mg/kg/day (b.i.d.) (A & E), 5 mg/kg/day (b.i.d.) (B & F), 10 mg/kg/day (b.i.d.) (C & G), where A-B-C from tail-flick and E-F-G from hot-plate. Antinociception was calculated as % of maximum possible effect, as described in *Methods*. The area under the curve analysis (AUC) is shown in D and H for tail-flick and hot-plate assays respectively. All data are represented as Mean ± SEM derived from n = 5-13 rats per group. Statistically significant differences between values of similar time-points within each graph, are shown as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001, produced from unpaired t-test with a Welch's correction. Additionally, significant differences between AUCs of day 1 and day 5 (or day 10) of the same morphine-treated group are shown as *p < 0.05, **p < 0.01 and ****p < 0.0001 and were calculated using unpaired t-test with a Welch's correction.

2.3.4. Overall dosing strategy affects tolerance development

The results up to this point suggested that the morphine dose determines the induction of antinociceptive tolerance. In addition, the data was interrogated to reveal if the time in a treatment paradigm when a specific dose is used, can also affect tolerance manifestation. Therefore, total antinociception (depicted as the area under the curve, AUC) over 5 consecutive days produced by the same morphine dose at 4 different treatment phases were compared using the tail flick (Fig. 11 A) and the hot plate assays (Fig. 11 B). The different time periods analysed were Phase 1 (1-5 days), Phase 2 (6-10 days), Phase 3 (11-15 days), Phase 4 (19-23 days) (For the 15 mg/kg/day dose the treatment range was extended to 23 days to compare the effects of 15 mg/kg/day morphine-treated animals in phase 2 (day 6-10); Fig. 12). The AUCs produced by the same dose of morphine was significantly different when this dose was used at different phases of the treatment protocol (Fig. 11 A, B). My results show that if the same dose was used at a later stage of the treatment paradigm, it resulted in less antinociception (Fig. 11 A, B). Thus, apart from the morphine dose itself, antinociceptive tolerance appears to depend on the overall dosing strategy.

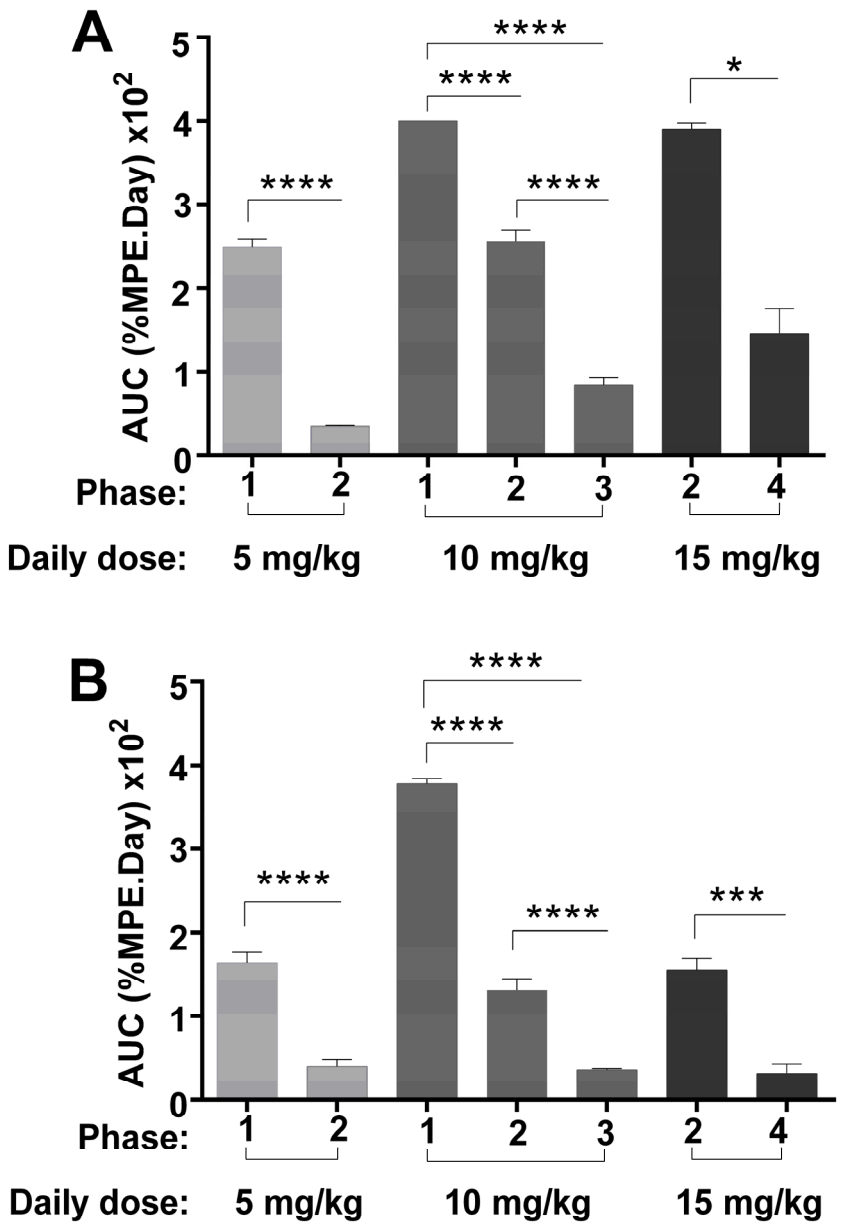


Figure 11. Effect of timing within a morphine dosing regimen on antinociceptive tolerance. The area under the curves (AUCs) of the antinociceptive effects of morphine in male Sprague Dawley rats 30 min post-administration (s.c.) were measured using the tail flick (A) and the hot plate (B) assays. Phases 1, 2, 3, 4 represent the treatment periods of 5 days as days 1-5, 6-10, 11-15 and 19-23 respectively with different repeated daily morphine administrations. AUCs of the same dose are compared against different phases of the treatment protocols. All data represent Mean ± SEM derived from n = 5-13 animals as described in Methods. Statistically significant differences between AUCs of different phases of treatment are shown as *p < 0.05, ***p < 0.001 and ****p < 0.0001 and were calculated using unpaired t-test with a Welch's correction.

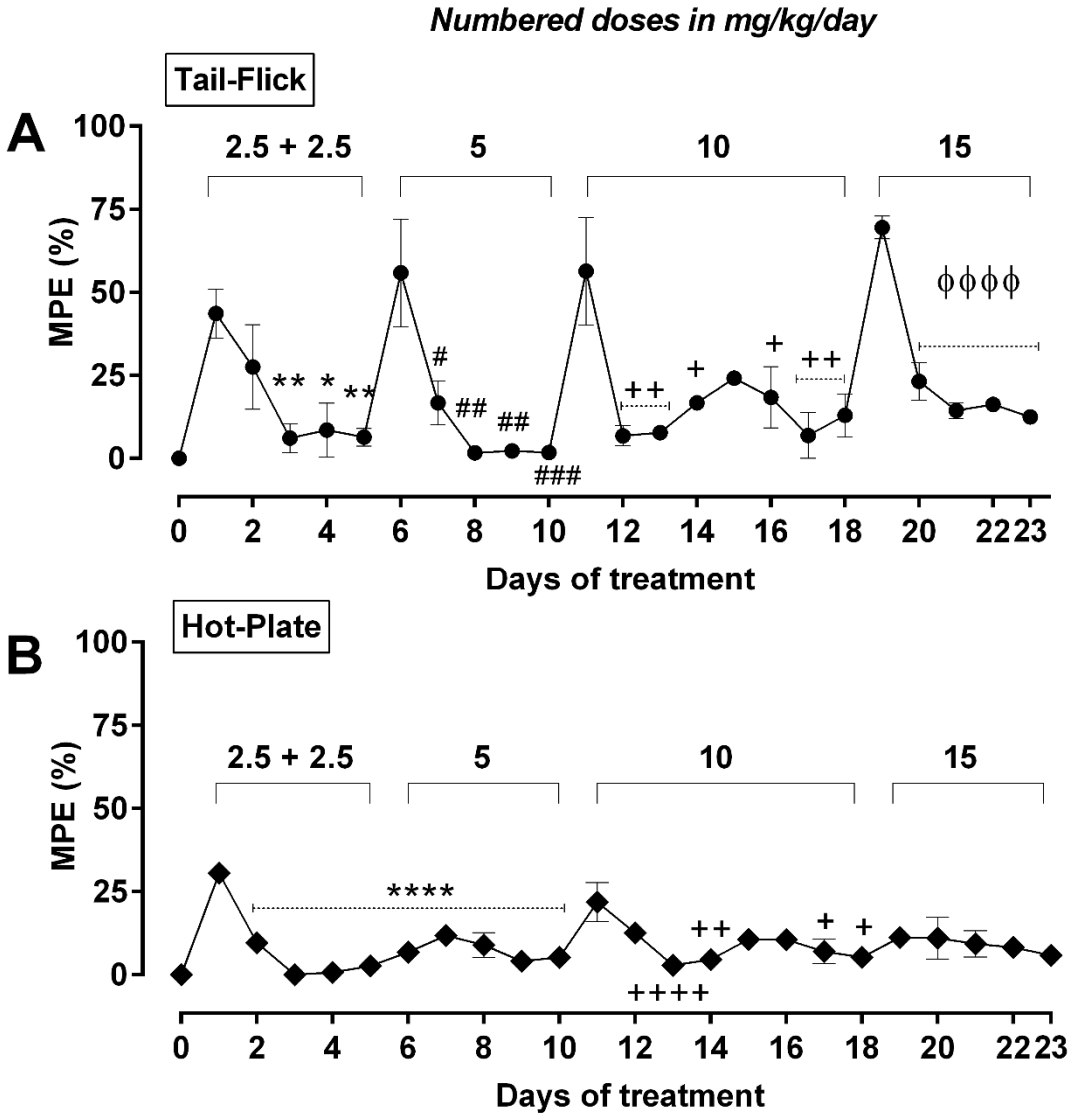


Figure 12. Long-term treatment with morphine: dose-dependent tolerance using tail-flick and hot plate assay. Antinociceptive effects of morphine were measured in male Sprague Dawley rats 30 min post-administration (s.c.) using the tail-flick (A) and hot plate (B) assays. Antinociception was calculated as the percentage of the maximum possible effect (MPE). Morphine was administered at multiple escalating doses: 2.5 (b.i.d.) → 5 → 10 → 15 mg/kg/day over 23 days, as described in Methods. All data are represented as Mean ± SEM derived from n = 5 rats. Statistically significant differences are shown as compared to day 1 (*p < 0.05, **p < 0.01 and ****p < 0.0001), against day 6 (#p < 0.05, ##p < 0.01, ###p < 0.001 and ####p < 0.0001), against day 11 (+p < 0.05, ++p < 0.01 and ++++p < 0.0001) or against day 19 (φφφφp < 0.0001), as produced from one-way ANOVA with Dunnett's multiple comparisons tests.

2.4. Discussion

The development of tolerance represents one of the major hurdles for effective clinical pain control using opioids. A vast number of studies since the ‘70s have investigated analgesic tolerance in rodents in terms of opioid exposure, molecular mechanisms involved, receptor expression and trafficking, neurophysiological parameters that are involved, etc. Nevertheless, the opioid tolerance issue is still a major concern in the clinic. Clinicians detect opioid tolerance by recording a significant drop of analgesia after repetitive administration to opioids, and a major part of their management strategy is to increase the dosing of the opioid to levels that will reconstitute sufficient analgesia or change the opioid drug to a more efficacious one. In the present study, I used two clinically-related markers of antinociceptive tolerance (the drop of antinociceptive efficacy and total antinociception) to investigate the effect of morphine’s dosing regimen in the manifestation of morphine tolerance.

Although different studies consistently reported the development of antinociceptive morphine tolerance within 3-8 days of repeated morphine administration (146,149,170,171,178,429), it is typically difficult to compare studies in the field due to different experimental set ups. In particular, choice of the assay and specific assay conditions (such as cut-off times), type of morphine (base, sulphate or hydrochloride salt), treatment protocols (dose, dosing frequency, continuous infusion, bolus injections), routes of administration, animal species and strains likely influence the experimental outcomes. Previous reports typically utilised only one test-method to assess morphine-induced tolerance. In contrast, two different thermal testing paradigms were employed in this study and distinct differences between the two assays with regards to the levels of antinociception and the timing of tolerance induction were observed. Since this approach allows to validate tolerance-induction in much more detail and reduces misinterpretation of antinociceptive responses. In this study, only recent and commercially

available equipment that can be accurately validated was utilised for the commonly employed radiant heat tail flick assay as well as the hot plate assay with widely used conditions (152,429,437,438). Tolerance was presented by two different methods to increase the robustness of my results: reduced efficacy of antinociception as % MPE and reduced total antinociception as AUCs. Using these indicators of tolerance, my results consistently suggest that morphine dosing (dose, frequency and increment) directly influences the development of tolerance. Under my experimental conditions, higher morphine starting doses or lower frequency not only delayed tolerance development but also reversed tolerance that was initiated with a lower starting dose or higher dosing frequency.

My results are supported by similar antinociceptive responses and tolerance profiles reported in earlier studies. Similar levels of morphine-induced antinociception (MPE%) were reported previously (167) and 10 mg/kg (b.i.d.) daily injections of morphine sulphate (s.c.) have been previously shown to result in a similar degree of tolerance in mice (140,141). Nevertheless, the data presented here indicate that a combination of factors such as treatment duration and general dosing strategy (e.g. dose, dosing frequency and dose increments) directly affects tolerance development and its manifestation. This is a finding which, to the best of my knowledge, has not been clearly reported in the literature to date, even though hints of evidence either supported or opposed this hypothesis. Previous studies provided some suggestions that dosing and frequency of dosing could affect the expression of tolerance. In one study a constant starting dose (0.5 mg/kg s.c.) was combined sequentially with different dose increments (+0.5 to 3 mg/kg s.c.) after mitigation of antinociception of the initial dose (179). This study also assessed the combination of different starting doses (0.5 to 3 mg/kg s.c.) with constant increments of dose (+1 mg/kg s.c.). In line with my results, in this short-term study higher starting doses and higher dose increments of morphine sulphate produced less tolerance in mice (179). Although

this was an acute study, it raised the question whether the same findings could be seen under chronic treatment conditions. A small number of studies that used 10 mg/kg/day (b.i.d.) morphine sulphate dosing reported earlier manifestation of antinociceptive tolerance (by a couple of days) compared to studies utilising a single-dose of 10 mg/kg/day morphine sulphate in rodents, but these differences may have easily occurred due to the large extent of differences in the protocols applied and species used (140,145,429). However, one single, recent and thorough study that contradicts my results associated mRNA changes of mu-opioid receptor splice variants with the induction of tolerance after long-term exposure to morphine (149). The authors provided ED_{50} data from a probit analysis of antinociception using assessed by a tail-flick assay in mice, using different dose-groups, and reported a lower tolerance manifestation in animals receiving low doses. Although the Xu *et al.* 2015 paper offers data with high power (high animal numbers and clinically-relevant long treatment periods), there are particular methodological disadvantages when using changes of ED_{50} values and shifts of dose-response curves in probit analysis as indicators of tolerance expression, that render these data difficult to interpret. Utilising an incremental exposure of morphine in already morphine-exposed animals of different treatment dose in order to produce a dose-response curve, produces an inevitable bias in response. For example, an animal that has been treated regularly with 40 mg/kg/day morphine, twice a day, for 21 consecutive days, is naturally expected to produce a reduced response when exposed to a much lower dose-screening range of morphine (1 to 10 mg/kg). In addition, the probit analysis used by a number of studies including the Xu *et al.* 2015 paper, is a less appropriate method than sigmoidal curves, due to their basic requirement of a binary outcome instead of the grading response offered by the tail-flick assay. Transforming grading responses to binary outcomes involves loss of information and therefore the analysis becomes suboptimal (439).

In summary, my data are consistent with all previous studies that antinociceptive tolerance to morphine is not a phenomenon where its expression and profile solely depends on the pharmacodynamics of the drug, but also on the dosing regimen employed. My study strengthened and expanded this hypothesis by providing solid evidence that higher doses of morphine not only lead to improved antinociceptive short-term relief (as total antinociception from a single-dose) and long-term relief (as total antinociception per treatment period) but also significantly delayed the induction of tolerance in terms of significant reduction in efficacy. Equally importantly, the results of this study on incremental strategies in the post-tolerant phase suggest that the higher the 'jump' of the second dosing level, the longer it takes the animal to develop those compensatory mechanisms needed for a low antinociceptive efficacy (tolerance). I acknowledge to the difficulty in discussing the clinical implications of these results given the strong adverse-effects associated with morphine use. I believe I provide substantial evidence in this study to support the hypothesis that the relationship between antinociceptive tolerance development and morphine is not just dependent on pharmacodynamics and receptor behaviour, but also on the magnitude of the starting dose and the frequency of exposure to morphine; two factors that have been largely underestimated in most pre-clinical studies investigating antinociceptive tolerance to date.

CHAPTER THREE

Profiling the effects of morphine dosing on motor behaviour

Preface to chapter three

Chapter two described how an adequate dosing strategy can delay antinociceptive tolerance and maintain effect antinociception during long-term treatment. In *chapter two*, I observed that a high starting and follow-up dose of morphine can maintain effective antinociception and reduce antinociceptive tolerance after chronic administration over a period of 2-3 weeks. The current study (*chapter three*) was designed to measure detailed locomotor activities, rearing and turning behaviours of morphine-treated animals in order to identify a possible connection between motor behaviour and antinociceptive tolerance.

3. Profiling the effects of morphine dosing on motor behaviour

Abstract

Efficient repetitive clinical use of morphine is limited by its numerous side effects, whereas analgesic tolerance necessitates subsequent increases in morphine dose to achieve adequate levels of analgesia. Although analgesic tolerance has been the focus of many studies, the effect of morphine dosing on non-analgesic effects has been overlooked. This study aimed to characterise development and progression of morphine-induced motor behaviour in relation to the development of antinociceptive tolerance. Adult male Sprague-Dawley rats were repetitively treated with subcutaneous morphine for 14 days in two dose groups (A: 5 mg/kg/day (b.i.d.) → 10 mg/kg/day; B: 10 mg/kg/day (b.i.d.) → 20 mg/kg/day). Motor behaviour was assessed daily (distance travelled, speed, moving-time, rearing, rotation) in an open-field arena, prior to and 30 min post-injections. Antinociception was measured using tail-flick and hot-plate assays. In both dosing groups, all measured parameters were highly suppressed on the first treatment day, followed by a gradual manifestation of behavioural tolerance as the treatment progressed. The kinetics of morphine-induced suppression of motor behaviour and subsequent behavioural tolerance were similar to those of antinociception and antinociceptive-tolerance. Animals of the high-dose group showed increased locomotor activity after 10 days of morphine treatment that paralleled antinociceptive tolerance. This excitatory phase converted to an inhibition of behaviour when a higher morphine dose was introduced. My results indicate that repetitive morphine dosing leads to broad tolerance against morphine-induced locomotor suppression in parallel with antinociceptive tolerance. I suggest that the excitatory locomotor effects of repetitive high-dose morphine exposure represent a signature of its antinociceptive tolerance.

Keywords: morphine dosing; behaviour; locomotor activity; antinociception; tolerance.

3.1. Introduction

Long-term clinical use of opioids such as morphine is limited due to its significant side-effects such drowsiness, itching, respiratory depression, constipation, addiction and dependence (111,112). Although predicting the appearance of morphine-induced side effects is important for effective pain-relief, the relationship between opioid dosing and the appearance of drug-induced side effects is currently not well established. In the clinic, pain-relief and side effects are different responses to morphine, which appear to correlate poorly (190). Behavioural side-effects of morphine in different clinical studies are both described as dose-dependent such as pruritus (250,440), as well as dose-independent, such as nausea and vomiting (251,440). In this context, it becomes important to understand how the dosing regimen can affect both morphine-induced antinociception, as well as the appearance of behavioural effects. Antinociceptive tolerance largely depends on treatment dose and dosing-protocol of morphine. One of our recent studies showed that a higher starting dose or larger increases of a testing dose of morphine produced overall less antinociceptive tolerance in rats (137).

The current literature shows inconsistent effects of morphine on animal behaviour. These inconsistencies are likely due to the use of different species/strains, routes of administration, types or formulations of morphine and differing treatment protocol (dose, frequency or duration of treatment) (192,194,441,442). Locomotor activity has been widely assessed to characterise behavioural effects of morphine-treated animals. While lower morphine-doses largely left locomotor activities unaffected, higher doses produced stimulatory or biphasic effects when morphine was administered acutely (191,193,441). Similarly, long-term morphine treatment with lower doses (1.25 to 5 mg/kg i.p.) showed no effects on locomotion, while higher doses (10 to 40 mg/kg i.p.) produced a biphasic effect with initial suppressive and subsequent increased locomotor activities (191). In addition, locomotor activity alone cannot reflect the

total behavioural side-effects profile of morphine, while concomitant measurement of locomotor activities together with other behavioural parameters is better suited to model the behavioural side-effects of morphine (193). In this study, 20 mg/kg of intraperitoneal morphine increased, while 10 mg/kg of morphine decreased horizontal movements in female mice, while both doses decreased rearing and grooming activities (193). In contrast, no dose-dependent differences in locomotion or rearing were detected in male rats in response to 0.5-50 µg intracerebroventricular morphine injections (441). These discrepancies illustrate that morphine-induced behavioural activities are, among other parameters such as route of administration and species, influenced by the gender of animals, which is not surprising as it is well described that female animals are more sensitive to morphine treatment (443,444). As such, female mice showed increased distance and rearing duration compared to male mice in response to morphine treatment (444). Moreover, different environmental settings have been used for behavioural tests. Especially changes to the illumination conditions, such as brightly illuminated (444,445,446), moderately illuminated (192,193), or low illuminated (191) have been reported. Since rodents are more active in the dark or under conditions of low illumination, brightly lit open-field arenas can distract the animals, which is likely to alter the results from experiments performed under conditions of low illumination intensity. In addition, most studies only tested the animal responses 30 min after acute treatment of morphine (192,193,441), which completely disregarded the known biphasic behavioural pattern of morphine exposure (191,447).

Although some studies combined several behavioural activities such as distance travelled, rearing, immobility or grooming after acute treatment of morphine (192,193,441), any possible connections between locomotion and other behavioural effects have not been established for repetitive long-term morphine treatment. Therefore, a significant gap of knowledge is evident

regarding the relationship between antinociceptive and behavioural effects of morphine, but also regarding its long-term effect on behaviour and antinociceptive tolerance. In this study, I measured multiple behavioural effects before, during and after long-term morphine treatment in rats. Animals were treated with two different morphine dosing-regimens to establish the influence of dosing regimen on behaviour and antinociceptive tolerance. Seven behavioural parameters were measured automatically in an open-field arena, which has been rarely done before (441). I aimed to generate a very detailed behavioural profile of long-term morphine treatment, behavioural tolerance and the influence of different morphine dosage-regimens, to accurately reflect existing relationships between behavioural and antinociceptive tolerance.

3.2. Methods

3.2.1. Animal maintenance and care

Twelve male Sprague-Dawley (SD) rats (233.6 ± 5.96 g, 8 weeks), supplied by the animal services of the University of Tasmania were housed as three littermates per cage at 22 ºC with 50-60 % humidity under an automated 12-hour day/night cycle (lights on at 7:00 am) with free access to food (Barastoc rodent cubes, Ridley Corporation, Melbourne, Australia) and water. Male rats were used to avoid any possible effects of the oestrous cycle of female rats (336). Moreover, all procedures and handling were approved by the University of Tasmania Animal Ethics Committee (A0013864) and were conducted according to *The Australian Code for the Care and Use of Animals for Scientific Purposes* (434). The experiments were also in compliance with the *ARRIVE* guidelines (435). F10SC veterinary disinfectant solution (Health and Hygiene Pty Ltd, Florida Hills, South Africa) was used for cleaning and hygiene purposes as a diluted solution (1:250 in water). Animals were handled for 5-6 days before the experiments were conducted. On the morning of behavioural tests, animals were transported to the testing room in their home cages and acclimatised to the test environment for 2 hours.

3.2.2. Treatment protocol

Body weight was recorded daily immediately prior to experiments in order to determine the accurate dosing for each rat. Commercially available 30 mg/ml morphine sulphate solution (Hameln Pharmaceuticals GmbH, Germany) was administered by daily subcutaneous injections between left thigh and the spinal cord. All animals were divided into two subgroups using a completely randomised design as previously described (448). Two sub-groups of animals received different morphine dosing-regimes. Group A (n = 6): morphine sulphate 5 mg/kg (twice daily) for 5 days, followed by a single dose (once daily) of 10 mg/kg from day 6 to 14. Group B (n = 6): morphine sulphate 10 mg/kg (twice daily) for 10 days, followed by a

single dose (once daily) of 20 mg/kg from day 11 to 14. Volumes of morphine solution for injection were ranged between 60 to 180 µl depending on the dose and body weights of the animals. For injection volumes of less than 60 µl, the volume was adjusted with sterile 0.9 % sodium chloride solution to fit into the volume range. Thesubcutaneous administration was previously shown to be an effective and quick route of morphine administration (449), with minimal discomfort to the animals. Different morphine doses and timing of injections were selected, based on our previous results (137). Morphine doses were doubled after the manifestation of antinociceptive tolerance induced by a starting dose. All doses were assessed for long-term safety in pilot trials before use in the present study. The illumination intensity of the laboratory was reduced prior to and during experiments to minimise discomfort to the animals. At the end of each study, animals were anesthetised with 5 % (w/v) isoflurane in oxygen at a flow rate of 1 L/min before decapitation.

3.2.3. Locomotor activity and other behavioural measurements

Behaviour was tested in an open-field arena in an automated Multi-Conditioning System (MCS) (TSE GmbH, Homburg, Germany) 2 min after nociception testing at pre-, post- (15, 30, 60, 120 and 180 min) administration of morphine over a period of 5 min on the 1st and the last treatment day of the same dose, since a 5 min observation period is widely used for open field measurements (192,193,446,450). On all other treatment days, the rats were tested only for baseline behaviour (pre-) and 30 min post-administration of morphine, which represents the time-point of morphine-induced maximal behavioural suppression on day 1. Baseline behaviour was defined as the basal behavioural measurements prior to morphine injection on each particular test-day. Rats were returned to their home cages after antinociceptive testing and kept there for 2 min before the start of behavioural measurements. Behavioural testing included seven different parameters (moving time, total distance travelled, speed, rotation

numbers, rearing numbers, rotation time and rearing time). Speed (m/s) was calculated as distance travelled (m) divided by the corresponding moving time (s) obtained from the system (MCS). Rotation numbers were calculated as the sum of clock-wise and counter-clockwise rotations obtained directly from the MCS. The area under the curves (AUC) was calculated by the trapezoid method using GraphPad Prism V6 software (GraphPad Software Inc., La Jolla, CA, USA).

The MCS included an internal noise/light/temperature insulation system and a 3D infrared-beam frame that provided fast and accurate animal movement detection (100Hz), combined with a high-resolution video monitoring and automated movement tracking. Quantification and visualisation of the MCS data were processed by the integrated system software (TSE ActiMot). The open-field arena was fully cleaned and dried between each animal. A background white noise (20 dB) generator was used during all experiments to cancel out any unexpected laboratory sounds.

3.2.4. Assessment of antinociception

Nociceptive thresholds were determined by two independent assays (tail-flick & hot plate) using commercially available tail-flick and hot/cold plate assay equipment (Ugo Basile, Comerio, Italy). Two independent assays were used to determine whether antinociceptive tolerance is dependent on the method of antinociception measurement. Animals were tested randomly to avoid any bias effects to due to multiple repeated measurements. The maximum exposure to the nociceptive thermal stimulus was 15 sec for the tail-flick (basal latency: 4.26 ± 0.22 sec) and 30 sec for the hot-plate assay (basal latency: 5.72 ± 0.29 sec). The infrared intensity of the tail-flick photocell was set at 30, whereas the plate temperature of the hot-plate was set at 54 ± 0.5 °C. The experimental settings for antinociceptive measurements used in this

study were previously described (137,152,437,438). Every rat was tested immediately prior to morphine administration as well as 15, 30, 60 and 120 min post-administration using both assays on the first and the last treatment day. Nociception measurements were conducted in a blinded manner and the mean of three independent measurements for each time-point with a 1 min interval between measurements was recorded to minimise 'handling' effects. The maximum possible effect (MPE) was defined as MPE % = 100 X [(test latency – baseline latency)/(cut-off time – baseline latency)] as previously described (436). Baseline latency was defined as the basal antinociceptive measurement prior to morphine injection on each particular test-day. The area under the curves (AUC) was calculated by the trapezoid method using GraphPad Prism V6 software (GraphPad Software Inc., La Jolla, CA, USA).3.2.5. Statistical analysis

Data are expressed as mean ± SEM and analysed by one-way ANOVA with Dunnett's multiple comparisons test or unpaired t-test, using GraphPad Prism v6 software (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons (Dunnett's test) were employed when F achieved $p < 0.05$ and there was no significant variance in homogeneity. A 'p' value less than 0.05 was considered statistically significant.

3.3. Results

3.3.1. Time-resolved efficacy of a single dose of morphine to locomotor behaviours: hypoactivity vs hyperactivity

To acquire a basic understanding of how repeated administration of morphine affects rat behaviours such as moving, rearing and rotation, rats were treated daily with 10 mg/kg (b.i.d.) morphine over a period of 10 days and their activities were recorded and assessed daily at regular intervals, for a total of 180 min after administration. The basal levels of activity (t = 0 min) at every day for the duration of treatment were similar with no significant differences, therefore no residual effects of morphine on the examined behaviour were observed at the beginning of every daily experiment from previous administrations. Behavioural scoring at every time-point was compared between days 1 and 10 for the recorded behaviours and their parameters (general locomotion Fig. 13; rearing and rotation Fig. 15). For general locomotion, at day 1 of morphine administration, suppression of locomotor activities was observed after 30 min at all parameters analysed (one-way ANOVA; $F(11, 46) = 12.43$; $p < 0.01$(distance); $F(11, 54) = 8.96$; $p < 0.0001$ (moving time); $F(11, 57) = 22.12$; $p < 0.001$ (speed)), which persisted until 60 min after administration (Fig. 13 A-C). The repression of all examined parameters of locomotion returned to their basal levels within 180 min after administration. However, after 10 days of daily repetitive administration of morphine (day 10), general locomotion is manifested as hyperactivity, as shown by significant increases in travelled distance (one-way ANOVA; $F(11, 46) = 12.43$; $p < 0.0001$) and moving speed (one-way ANOVA; $F(11, 57) = 22.12$; $p < 0.0001$), along with non-significant differences in moving time from basal levels. This change in the activity profile towards hyperactivity was accompanied by a shift of its time-peak at 15 min after morphine administration and a faster recovery to basal within 180 min of treatment (Fig. 13 A-C). Relevant behavioural activities induced by morphine 5 mg/kg (b.i.d.) for 5 days repeated treatment are shown in Fig. 14.

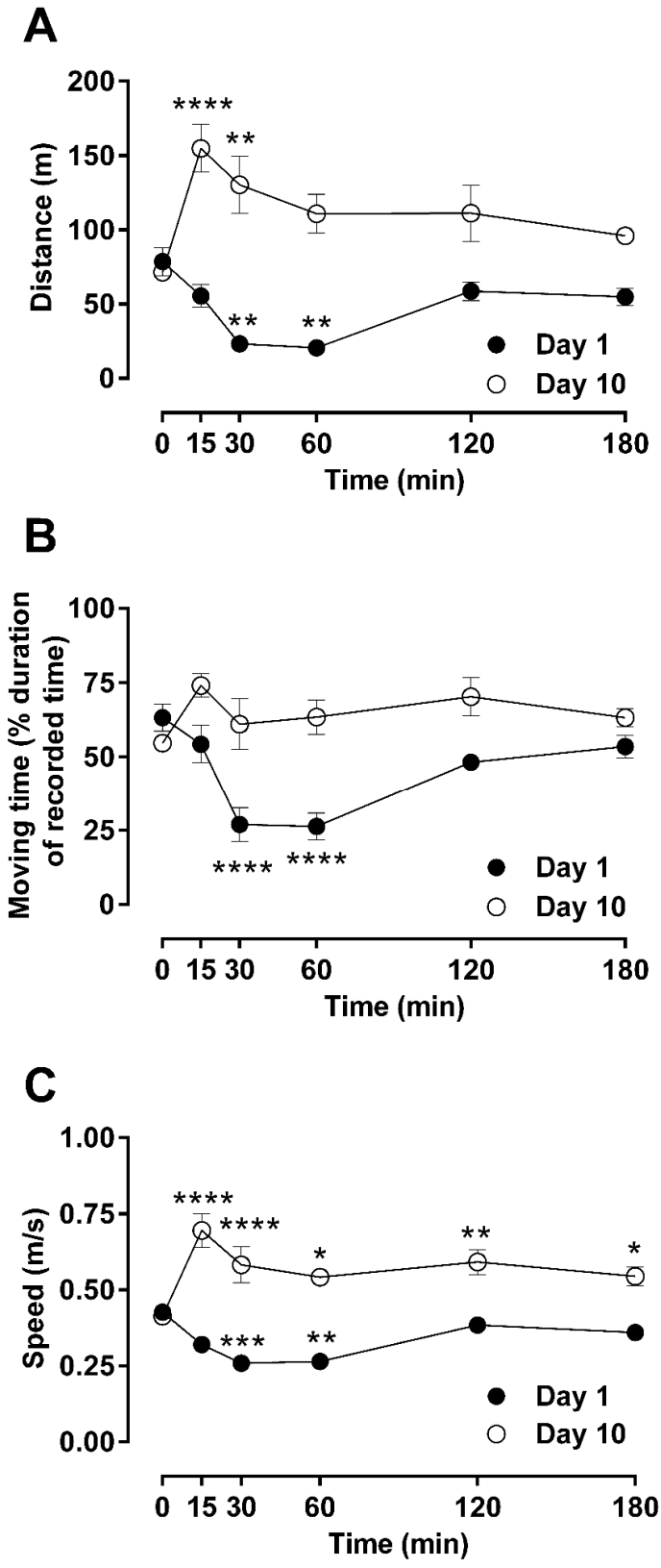


Figure 13. Time-resolved locomotor activities after repeated morphine treatment. Locomotor parameters recorded in an open-field arena after a single subcutaneous injection of morphine (10 mg/kg, b.i.d. over 10 days) on day 1 or day 10 in male Sprague Dawley rats. Motor behaviour of treated animals was assessed by quantification of total distance travelled (A), total moving duration (B) or average speed (C), for a period of 180 min post-administration. Statistically significance ($p < 0.05$) against the basal effects of day 1 is shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ and was calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values are presented as mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

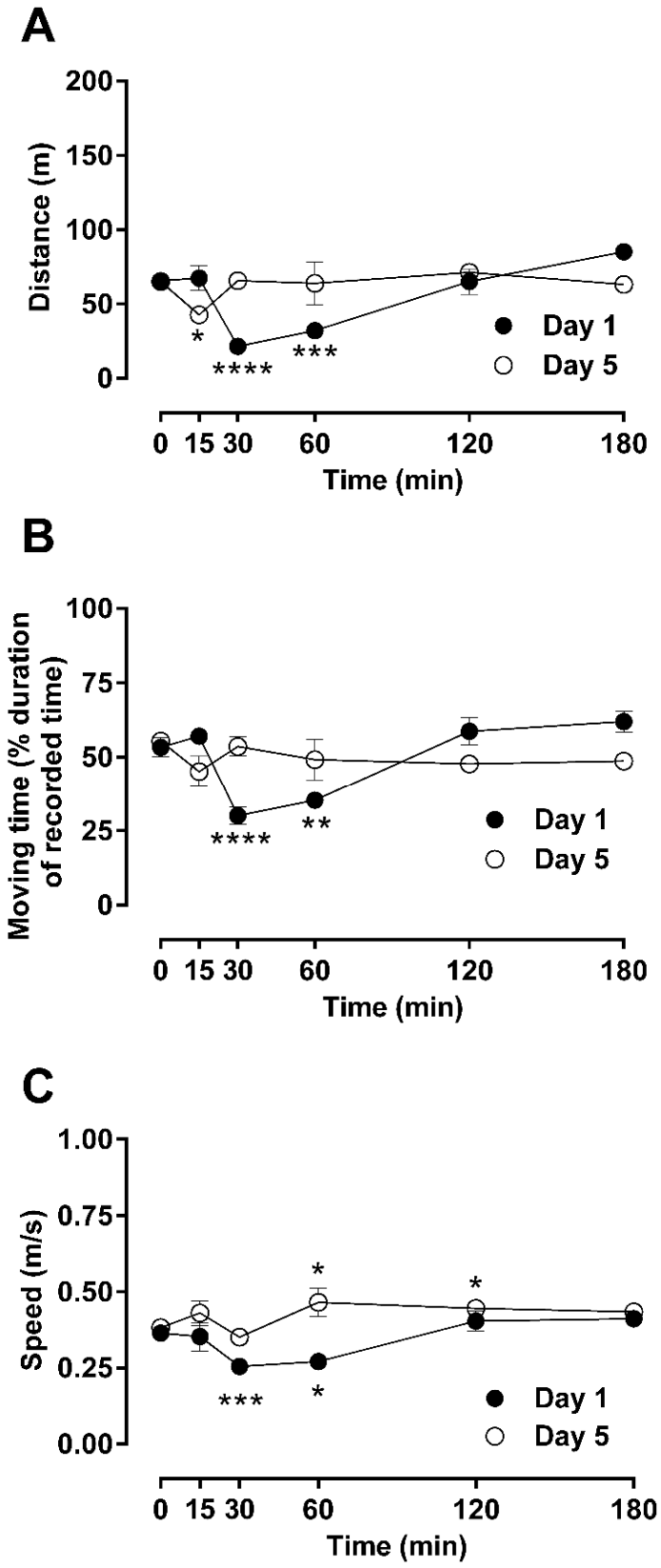


Figure 14. Time-resolved locomotor activities after repeated morphine treatment. Open-field locomotor activities after a single subcutaneous injection of morphine (5 mg/kg, b.i.d. over 5 days) on day 1 or day 5 were measured in male Sprague Dawley rats. Activities of treated animals were measured as distance travelled (A), moving time (B), or speed of movement (C) over a period of 180 min. Statistically significance ($p < 0.05$) against the basal effects of day 1 is shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ and was calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values are presented as mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

The specific locomotor behaviours of rotation and rearing were also analysed in a similar time-resolved manner in terms of total score (numbers; Fig. 15 A & B) and duration (time; Fig. 15 C & D). Rotational behaviour was suppressed by morphine at day 1 at a similar fashion as general locomotion in terms of peak and recovery timing (one-way ANOVA; $F(11, 42) = 31.56$; $p < 0.05$) (Fig. 15 A, C). As in the case of general locomotion, the rotational behaviour was also observed to be significantly increased at day 10 of treatment compared to basal (one-way ANOVA; $F(11, 42) = 31.56$; $p < 0.01$) (Fig. 15 A, C). Interestingly, although rearing showed to be significantly suppressed by morphine at day 1 similarly to general locomotion (one-way ANOVA; $F(11, 39) = 45.85$; $p < 0.0001$), this behaviour remained suppressed at day 10 of treatment without contributing to the hyperactivity profile usually seen in the previously recorded parameters at day 10 (Fig. 15 B & D). The corresponding behavioural activities induced by morphine 5 mg/kg (b.i.d.) for 5 days repeated treatment are shown in Fig. 16.

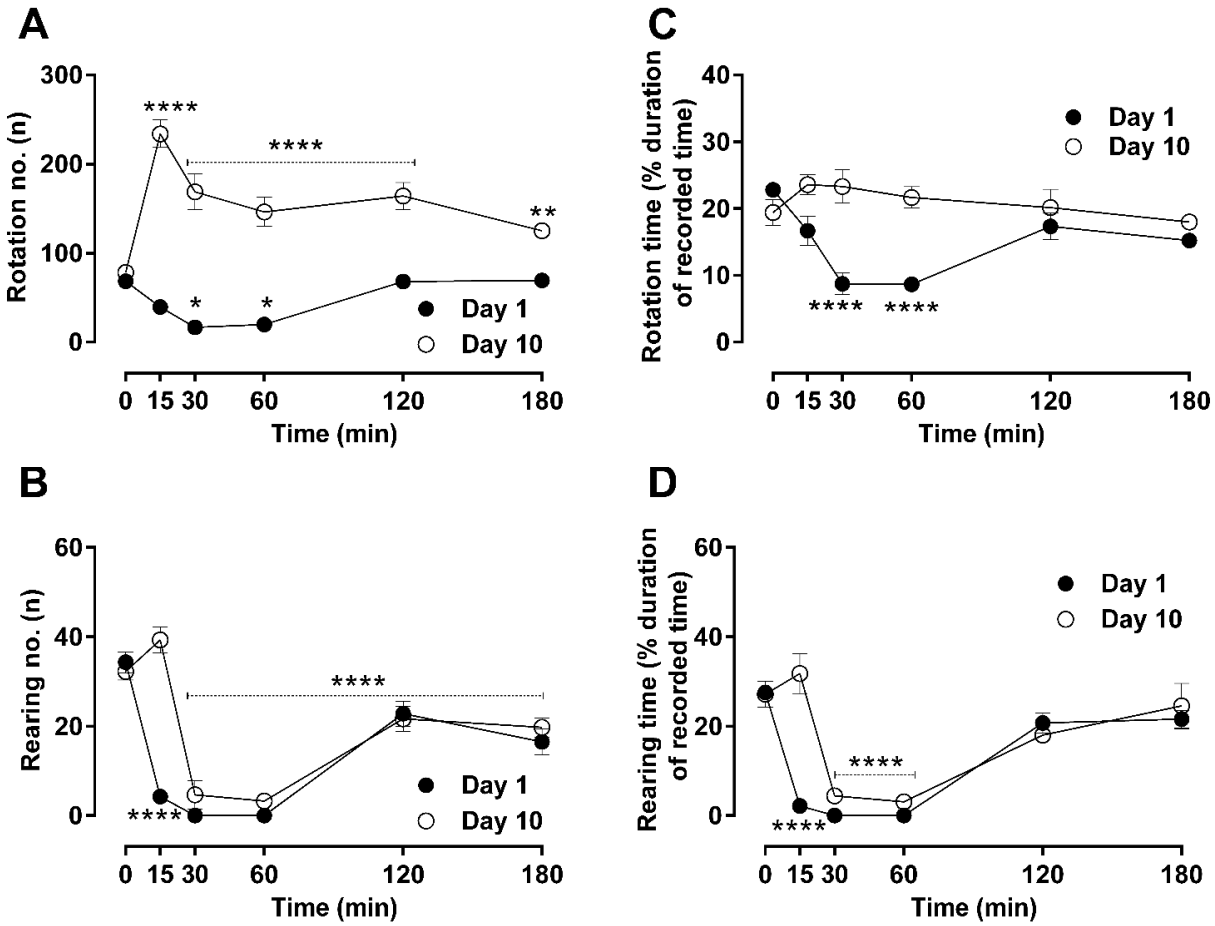


Figure 15. **Time-resolved rotation and rearing activities after repeated morphine treatment.** Open-field turning and rearing activities after a single subcutaneous injection of morphine (10 mg/kg, b.i.d. over 10 days) on day 1 or day 10 were measured in male Sprague Dawley rats. Activities of treated animals were measured as rotation no (A), rearing no (B), rotation time (C) and rearing time (D) over a period of 180 min. Statistically significance ($p < 0.05$) against the basal effects of day 1 is shown as *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ and was calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values are presented as mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

To evaluate the overall behavioural effects of repeated morphine administration over a period of 10 days, the overall scores of Fig. 13 and Fig. 15 were quantified as area under the curves (AUC) and presented in Table 15. From this analysis, it is evident that morphine significantly stimulates locomotion after 10 days of repetitive administration, compared to day 1 where it significantly suppresses locomotion, with the notable exception of rearing behaviour that persists to be suppressed at day 10 (Table 15).

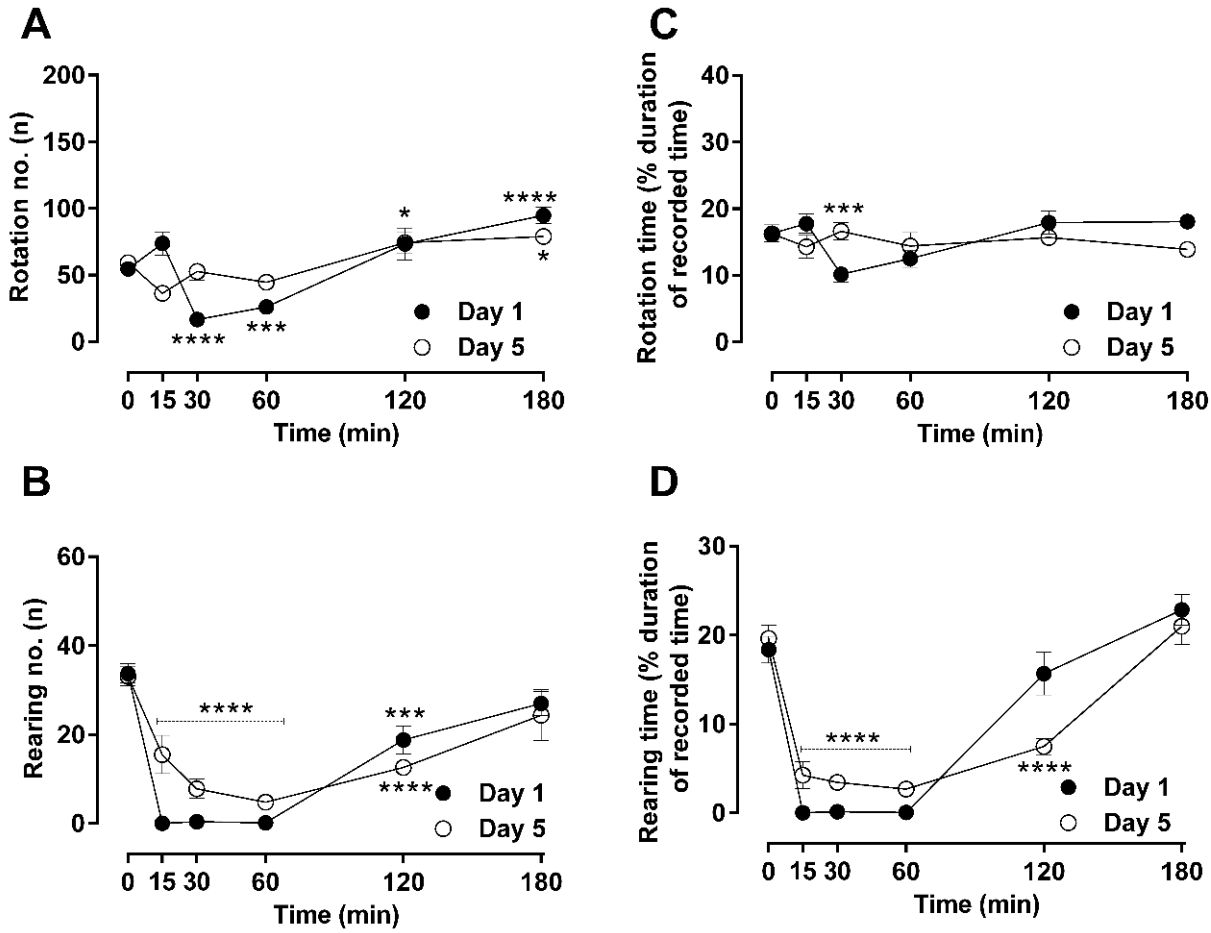


Figure 16. Time-resolved rotation and rearing activities after repeated morphine treatment. Open-field turning and rearing activities after a single subcutaneous injection of morphine (5 mg/kg, b.i.d. over 5 days) on day 1 or day 5 were measured in male Sprague Dawley rats. Activities of treated animals were measured as rotation no (A), rearing no (B), rotation time (C) and rearing time (D) over a period of 180 min. Statistically significance ($p < 0.05$) against the basal effects of day 1 is shown as *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ and was calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values are presented as mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

Table 15. Overall comparison of motor-behaviours from day 1 after repeated morphine treatment. Total scoring of motor-behaviours recorded over 180 min after subcutaneous administration of morphine 10 mg/kg (b.i.d.) over 10 days repeated treatment. Parameters (presented as area under curve; AUC) were calculated from the behavioural curves in Figures 13 and 14. Statistically significant differences between AUCs of each behaviour were assessed using an unpaired t-test.

Behavioural Parameter	**AUC units**	**Day 1** ***(Dose-group B)***	**Day 10** ***(Dose-group B)***	**Significant difference**
Distance	min * days	8958 ± 995	19492 ± 1562	t (10) = 5.688; p <0.001
Moving time	% of recorded time * days	8349 ± 643	11715 ± 643	t (9) = 3.668; p < 0.01
Speed	(metre/sec) * days	60.22 ± 1.25	99.51 ± 3.34	t (10) = 11.02; p < 0.0001
Rotation numbers	incidences * days	8374 ± 550	25423 ± 1761	t (10) = 9.24; p < 0.0001
Rotation time	% of recorded time * days	2464 ± 240	3704 ± 199	t (10) = 3.979; p < 0.01
Rearing numbers	incidences * days	3018 ± 308	3717 ± 206	t (8) = 1.887; p = 0.096
Rearing time	% of recorded time * days	2116 ± 171	2803 ± 458	t (9) = 1.297; p = 0.2269

3.3.2. Dose-dependent efficacy of repetitive morphine administration and incremental changes of dosing on locomotor behaviour: hypoactivity vs hyperactivity

To investigate the effect of morphine dosing regimens on locomotion and related behaviour, the animals were treated with two dosing regimens that differ in morphine's dose and duration of treatment (5 mg/kg/day b.i.d. or 10 mg/kg/day b.i.d.), followed by a subsequent change in administration of morphine (double dosing in single daily injections) until day 14 of treatment (Fig. 17). Noticeably, no differences between the basal locomotion, rotation or rearing activities over the total treatment-period of 14 days were observed, which were recorded daily (every morning) immediately before morphine injections (Fig. 18 and 19).

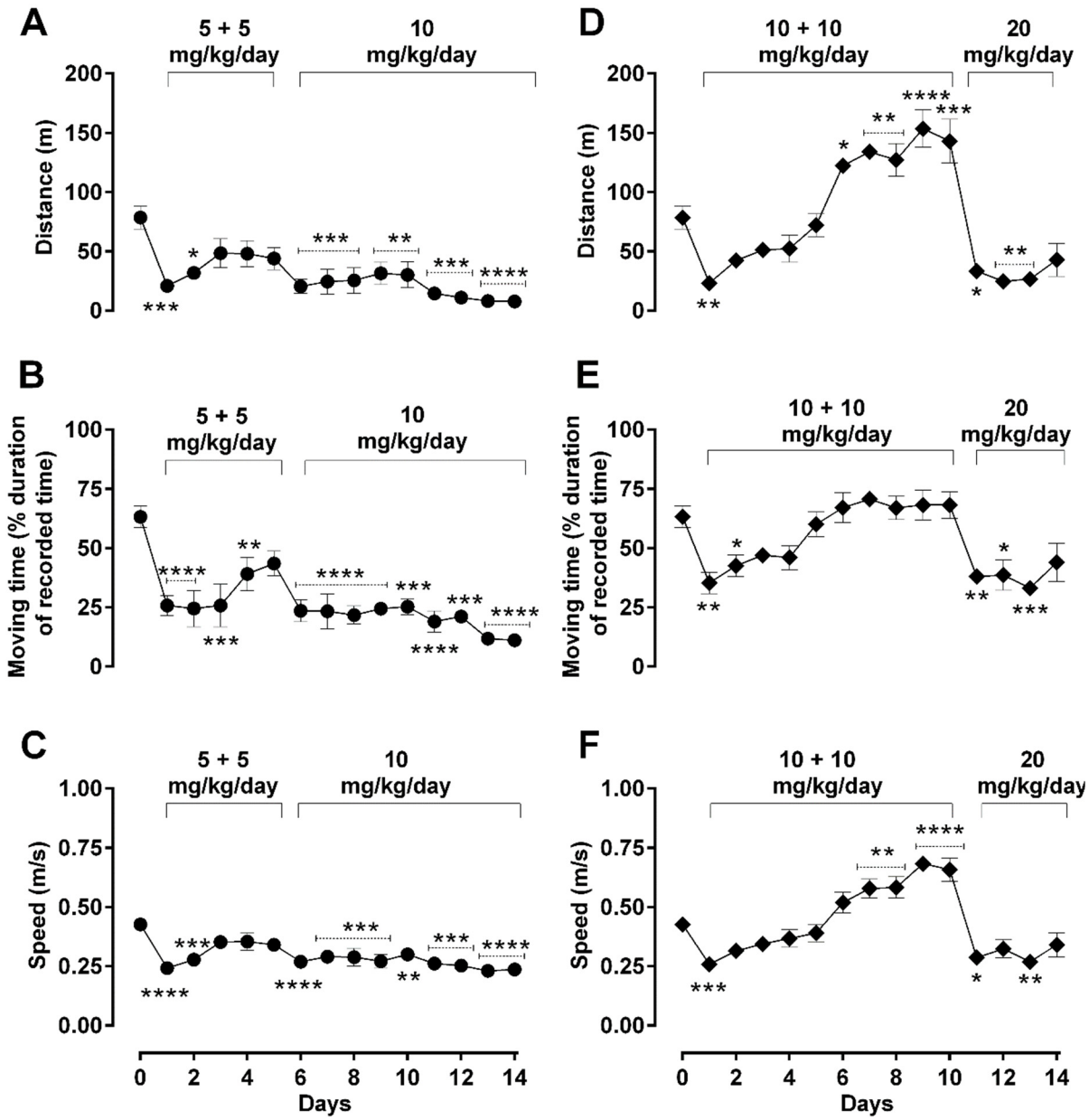


Figure 17. Dose-dependent locomotor activities after long-term morphine treatment. Locomotor parameters recorded in an open-field arena after daily subcutaneous injections of morphine in male Sprague Dawley rats, at a daily 30 min mark post-administration during a 5 min recording period. The motor behaviour of treated animals was assessed by quantification of distance travelled (A, D), moving duration (B, E) or average speed (C, F). Two morphine regimens were used in two different groups of animals respectively: 5 (b.i.d.) → 10 mg/kg (A, B, C) and 10 (b.i.d.) → 20 mg/kg (D, E, F) over a total period 14 days, as described in *Methods*. Values are presented as mean ± SEM (n = 6 animals per group). Statistically significant ($p < 0.05$) differences compared against day 0 are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Error bars are present in all graphs but are sometimes too small to be visible.

In the ‘low’ starting dose treatment group (5 mg/kg b.i.d. → 10 mg/kg/day; Fig. 17 A), a significantly reduced moving distance was observed at days 1 and 2 (one-way ANOVA; $F(14, 48) = 4.26$; $p < 0.05$), with a slow but steady recovery till day 5. The subsequent change in the method of administration of morphine (from twice daily 5mg/kg to once daily 10mg/kg) on day 6 onwards somewhat increased the suppressive effect of morphine on moving distance returning the difference from basal back to significant levels (one-way ANOVA; $F(14, 48) = 4.26$; $p < 0.001$). No behavioural recovery was observed from day 6 to the end of treatment period (day 14). The parameter of moving time (Fig. 17 B) and moving speed (Fig. 17 C) expressed very similar responses to morphine treatment and changes in administration, as in the case of total distance.

In the second group of ‘high’ morphine dosing (10mg/kg/day b.i.d. till day 10 → 20 mg/kg/day till day 14), the same locomotive parameters were assessed, with a marked change in the overall profile manifested (Fig. 17 D-F). In these animals, the 10 mg/kg/day b.i.d. morphine significantly reduced moving distance at day 1 compared to basal (one-way ANOVA; $F(14, 47) = 19.20$; $p < 0.01$), however, the recovery to basal values was not only at a fast and steady rate despite repetitive morphine administration, but also progressed to a significant increase in travelled distance scoring compared to basal, from day 6 (one-way ANOVA; $F(14, 47) = 19.20$; $p < 0.05$) until it reached a hyperactivity plateau at day 10. When morphine administration changed to a single dose of 20 mg/kg/day from day 11 (Fig. 17 D), the suppressive effect of morphine returned to the observed levels of day 1 (one-way ANOVA; $F(14, 47) = 19.20$; $p < 0.05$) and remained suppressed until the end of treatment period (day 14). This pattern of morphine-induced changes observed for travelled distance was also replicated to the rest of observed parameters of general locomotion (moving time; Fig. 17 E and speed of movement; Fig. 17 F), as well as rotational behaviour (Fig. 20 A & 20 C).

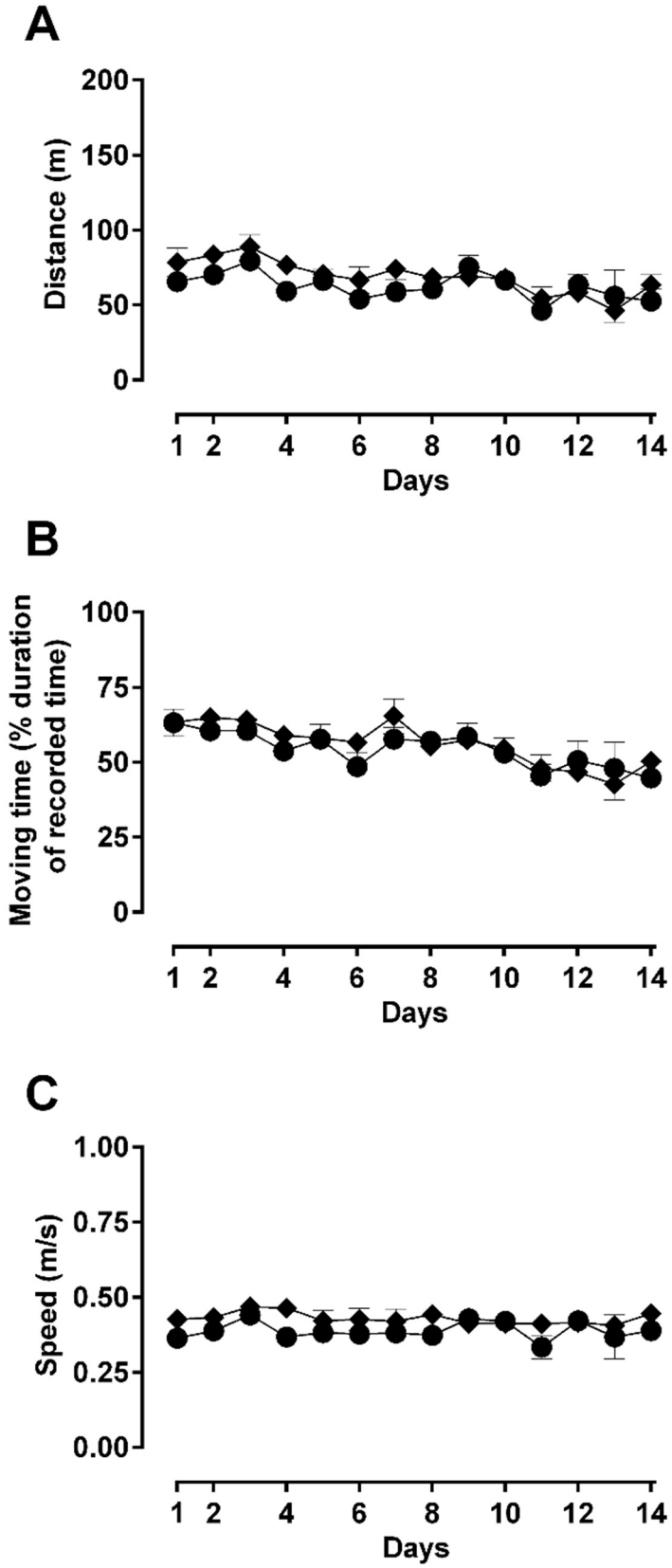


Figure 18. Basal locomotor activities of rats. Open-field locomotor activities at pre-administration (basal) in male Sprague Dawley rats over a period of 14 days. Activities of animals were measured as distance travelled (A), moving time (B), or speed of movement (C). Values are presented as mean ± SEM (n = 6 animals per group). No statistically significant differences were observed against day 1 using one-way ANOVA with Dunnett's multiple comparisons tests. Error bars are present in all graphs but are sometimes too small to be visible.

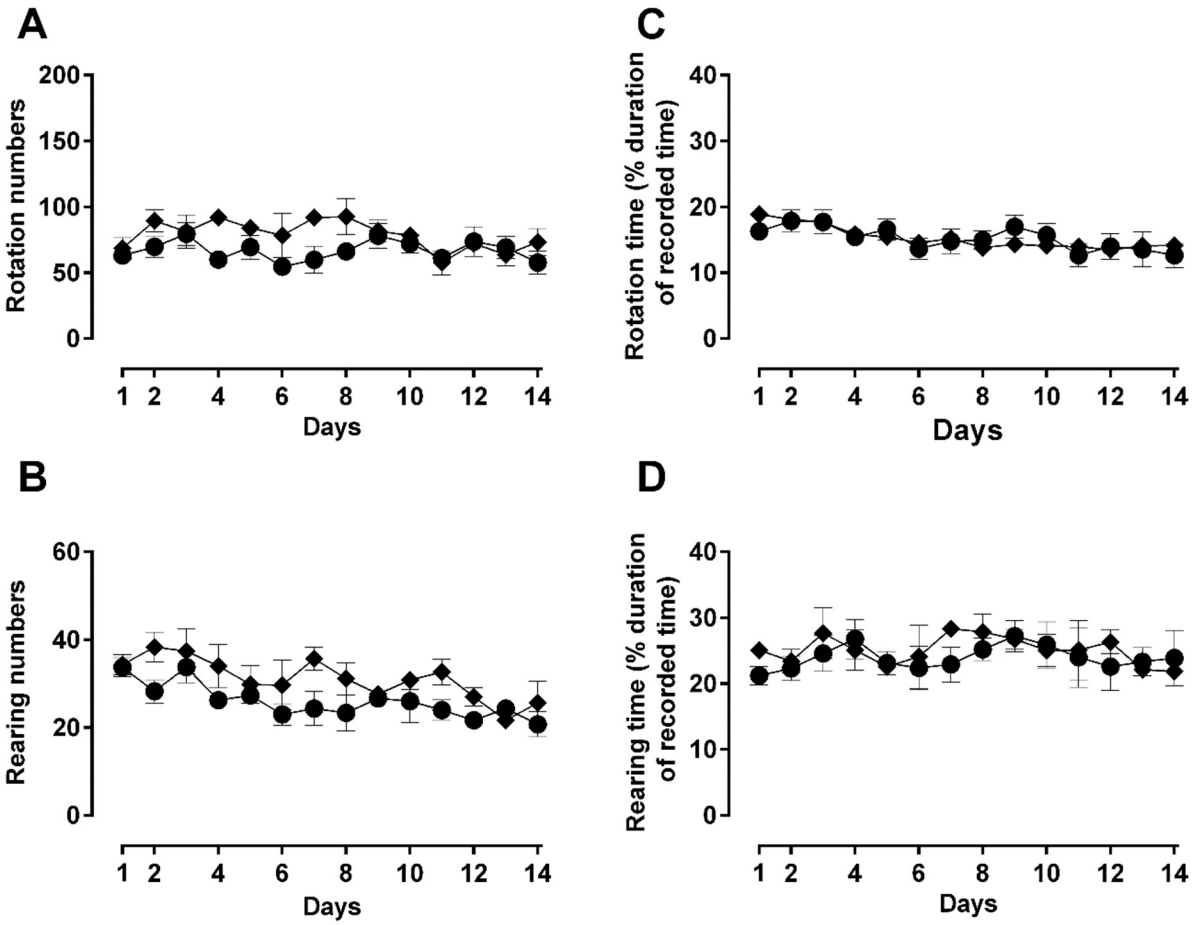


Figure 19. Basal rotation and rearing activities of rats. Open-field rotation and rearing activities at pre-administration (basal) in male Sprague Dawley rats over a period of 14 days. Activities of animals were measured as rotation no (A), rearing no (B), rotation time (C) and rearing time (D). Values are presented as mean ± SEM (n = 6 animals per group). No statistically significant differences were observed against day 1 using one-way ANOVA with Dunnett's multiple comparisons tests. Error bars are present in all graphs but are sometimes too small to be visible.

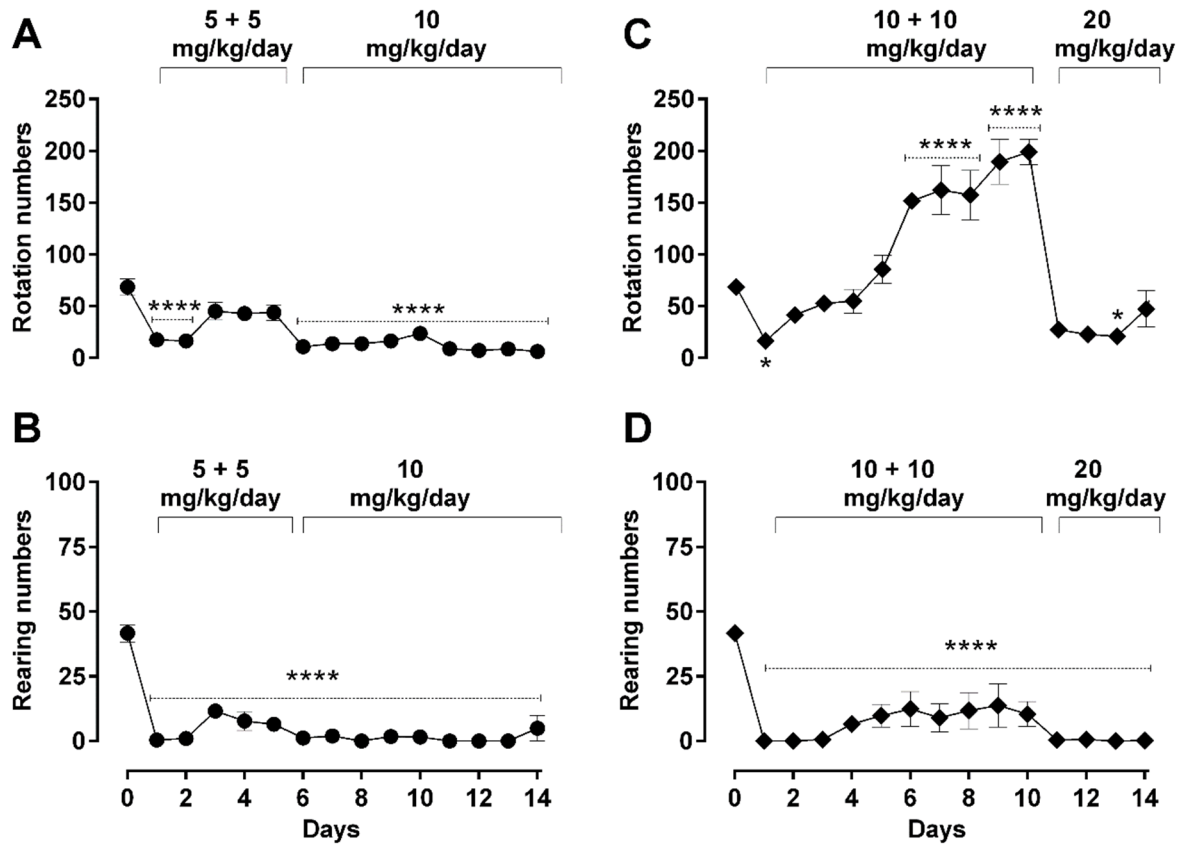


Figure 20. Dose-dependent rotation and rearing activities after long-term morphine treatment. Rotation and rearing behaviours recorded in an open-field arena after daily subcutaneous injections of morphine in male Sprague Dawley rats, at a daily 30 min mark post-administration during a 5 min recording period. Activities of treated animals were measured as rotation numbers (A, C), and rearing numbers (B, D). Two morphine regimens were used in two different groups of animals respectively: 5 (b.i.d.) $\rightarrow$ 10 mg/kg (A, B) and 10 (b.i.d.) $\rightarrow$ 20 mg/kg (C, D) over a total period 14 days, as described in *Methods*. Values are presented as mean ± SEM (n = 6 animals per group). Statistically significant ($p < 0.05$) differences compared against day 0 are shown as *$p < 0.05$ and ****$p < 0.0001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Error bars are present in all graphs but are sometimes too small to be visible.

In the case of rearing behaviour, data showed a similar profile in morphine's effect during repetitive administration for both 'low' and 'high' dose groups (Fig. 20 B & 20 D). Morphine significantly suppressed rearing from day 1 until day 14 (one-way ANOVA; $F(14, 53) = 35.57$; $p < 0.0001$) without any recovery or observed increase in recorded activity, even when a change in morphine's administration from twice daily to single double-dose daily (5mg/kg/day b.i.d. → 10m/kg/day; Fig. 20 B, 10 mg/kg/day b.i.d. → 20 mg/kg/day; Fig. 20 D), essentially staying suppressed until the entire treatment period.

The regimen-dependent behavioural changes were also calculated as area under the curves (AUCs) over the whole treatment period of 14 days and comparison between groups is shown in Table 16. The AUCs of behavioural parameters of the higher dosing paradigm (10 mg/kg/day b.i.d. → 20 mg/kg/day), were significantly higher than the AUCs of the lower treatment paradigm (5 mg/kg/day b.i.d. → 10 mg/kg/day), with the exception of rearing numbers and rearing time (Table 16). Thus morphine 10 mg/kg (b.i.d.) → 20 mg/kg/day treated animals showed more locomotor and rotational behavioural changes (hyperactivity) than morphine 5 mg/kg (b.i.d.) → 10 mg/kg/day group (Table 16).

Table 16. Overall comparison of motor behaviours between different dosing groups. Total scoring of recorded motor behaviours (presented as area under the curve; AUC) were calculated from the behavioural curves in Figures 17 and 20. Statistically significant differences between AUCs of each behaviour were assessed using an unpaired t-test.

Behavioural Parameter	**AUC units**	**Dose group A: *Morphine 5 mg/kg (b.i.d.) → 10 mg/kg/day***	**Dose group B: *Morphine 10 mg/kg (b.i.d.) → 20 mg/kg/day***	**Significant difference**
Distance	min * days	369.6 ± 71.6	982.3 ± 134.3	$t(9) = 3.785$; $p < 0.01$
Moving time	% of recorded time * days	305.5 ± 32.6	700.2 ± 51.4	$t(9) = 6.169$; $p < 0.001$
Speed	(metre/sec) * days	3.80 ± 0.23	5.69 ± 0.42	$t(9) = 3.702$; $p < 0.01$
Rotation numbers	incidences * days	255.8 ± 48.4	1200 ± 124.2	$t(9) = 6.552$; $p < 0.001$
Rotation time	% of recorded time * days	103.1 ± 12.6	233.4 ± 22.2	$t(9) = 4.821$; $p < 0.001$
Rearing numbers	incidences * days	48.0 ± 15.5	97.4 ± 30.1	$t(10) = 1.462$; $p = 0.174$
Rearing time	% of recorded time * days	19.4 ± 2.86	40.6 ± 9.9	$t(8) = 1.690$; $p = 0.130$

3.3.3. The relationship between antinociceptive tolerance and locomotor activities

To better understanding of the clinical significance of morphine's biphasic behavioural effect on locomotor behaviour between different dosing regimens, I aimed to compare these effects with morphine's major pharmacological drawbacks, antinociceptive tolerance. Antinociception was measured using two major assays (tail-flick and hot-plate) and tolerance was defined as a significant reduction of antinociceptive efficacy, whereas distance travelled was measured using open-field test over a period of 180 minutes after injections at day 5 or day 10 as described in *Methods*. The area under the curves (AUC) of the first treatment day (day 1) and day 5 for Group A (5 mg/kg/day b.i.d.) or day 10 for Group B (10 mg/kg/day b.i.d.) were compared using unpaired t-test (Fig. 21). Animals in the first morphine dose group (5 mg/kg b.i.d.) showed no difference in distance travelled between days 1 and 5, although they exhibited significant antinociceptive tolerance in both assays used (tail-flick; unpaired t-test; $t(10) = 8.48$; $p < 0.0001$ and hot-plate; unpaired t-test; $t(10) = 9.80$; $p < 0.0001$, as shown in (Fig. 21 A)). However, animals in the larger morphine dose group (10 mg/kg b.i.d.), presented hyperactivity (e.g. significantly higher locomotion) at day 10 when antinociceptive tolerance was first manifested in these animals, compared to day 1 (unpaired t-test; $t(7) = 7.04$; $p < 0.001$) as shown in Fig. 21 B. These data show collectively that antinociceptive tolerance due to repetitive morphine administration is an independent effect of morphine's effects on locomotor behaviour, which profile is largely dependent on the dosage regimen used.

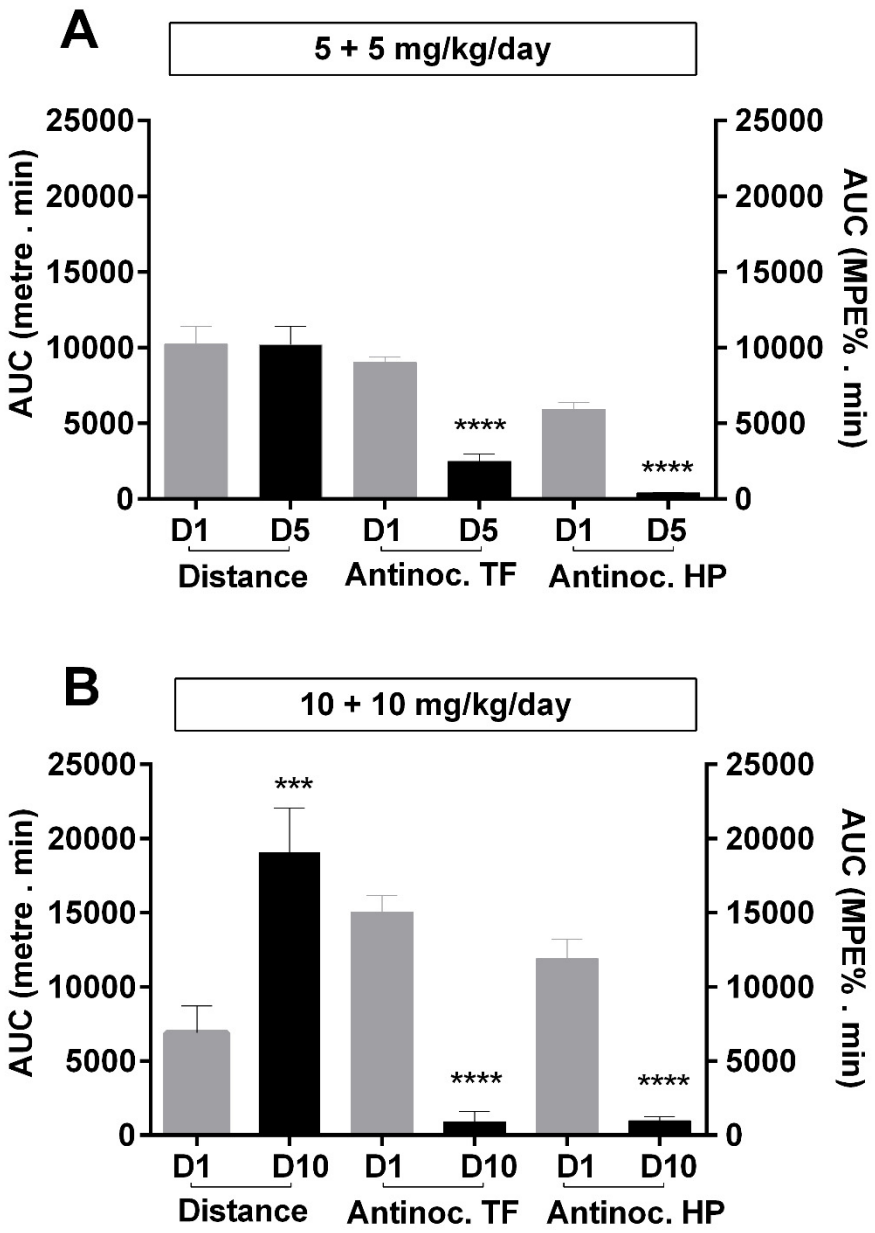


Figure 21. Overall behavioural and antinociceptive effects after long-term morphine treatment. The area under the curves (AUC) of distance travelled and antinociception in male Sprague Dawley rats over a period of 180 min post-administration of subcutaneous morphine at different days through a treatment period. The curves used to calculate these AUC values are shown in the Fig. 13 A, Fig. 15 A and Fig. 22. Morphine was administered daily as 5 mg/kg (b.i.d.) (A) over 5 days or 10 mg/kg b.i.d. (B) over 10 days. D1, D5 or D10 represents day 1, day 5 or day 10 respectively. Antinociception was assessed by two assays, tail-flick assay (TF) and hot-plate assay (HP). Values are presented as mean ± SEM (n = 6 animals per group). Statistically significant ($p < 0.05$) differences from day 1 of every treatment groups are shown as ****$p < 0.0001$ and were calculated using unpaired t-test. Error bars are present in all graphs but are sometimes too small to be visible.

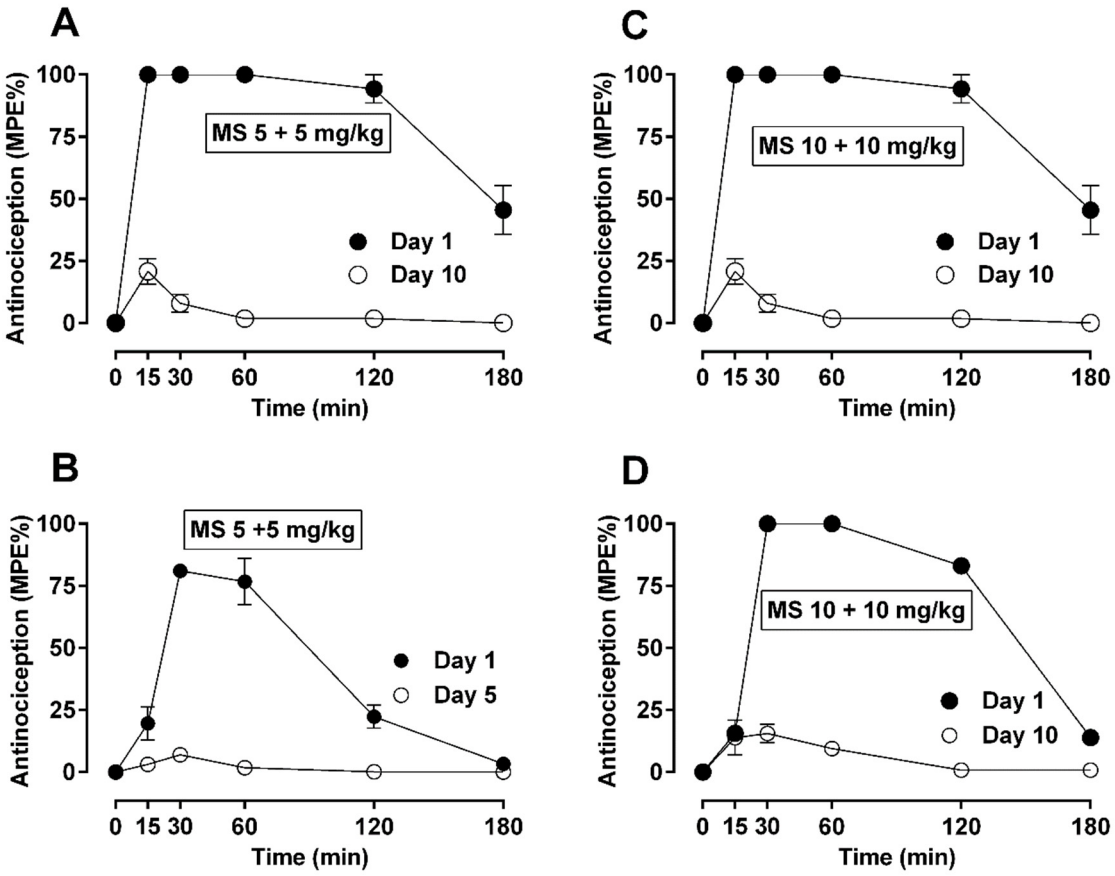


Figure 22. Antinociceptive effects of daily morphine-treated rats. Antinociceptive effects of 5 mg/kg (b.i.d.) and 10 mg/kg (b.i.d.) morphine-treated Sprague Dawley rats (n = 6 per group) over the course of 5 to 10 days (groups A and B). The antinociceptive effects were measured using tail-flick (A, C) and hot-plate (B, D) assays. The area under the curves are shown in Fig. 21. Error bars are present in all graphs but are sometimes too small to be visible.

3.4. Discussion

Morphine is the gold-standard for the treatment of chronic or cancer pain. Nevertheless, long-term use of morphine is severely limited by its biphasic effects on motor behaviour (inhibitory or excitatory) and the manifestation of analgesic tolerance (e.g. reduced analgesic efficacy). Despite an increased knowledge about morphine's activities, little is known how its dosing regimens affect the manifestation of motor effects and analgesic tolerance.

Euphoria, lethargy or drowsiness are very common clinical side-effects of morphine (451,452,453) and are mirrored by the hypoactivity reported in rodents administered with morphine (454). The connection between morphine dosing and the appearance of these behavioural effects has been investigated over several decades with different experimental setups and paradigms. In contrast to the present report, a previous study measured horizontal movement using male Holtzman albino rats after intraperitoneal morphine injections over a 1 or 2 hour observation period in their home cages over a total period of up to 4-8 hours (191). However, animal behaviour is largely governed by an interest in their surroundings. Therefore, if animals are tested in a familiar environment, such as their home cage, as opposed to an open filed arena, the reduced novelty will result in reduced exploratory behaviour and therefore locomotion (455). In addition, since this study did not measure detailed time points over the first hour after morphine injection that coincides with its maximum antinociceptive effect, the results of this study cannot be directly compared to the present study and are also unable to connect the behavioural effects of morphine with its antinociceptive effects, which was the aim of the present study (191).

The relationship between the behavioural effects of morphine, antinociceptive tolerance and morphine dosing has been elusive, mainly due to reported inconsistencies in experimental results, which are likely the consequence of different experimental approaches with regards to route of administration, type or formulation of morphine, type of animals used as well as treatment protocols (dose, frequency or duration of treatment) (192,194,441,442). All these experimental variables are likely to influence the effect of morphine on motor behaviour and therefore highlight the need for a study that examined the relationship between morphine's behavioural effects and dosing in a unified model. Understanding how morphine's dosing contributes to behaviour is crucial for future clinical strategies to reduce morphine's side-effects.

I assessed hypoactivity by monitoring the major parameters of locomotor activity (distance, speed, moving-time, rotational behaviour and rearing), which can be seen as surrogate markers for morphine-induced motor side-effects. Locomotor activity was previously used to assess behavioural side-effects after acute or chronic treatment of morphine (191,193,194). However, locomotion alone cannot represent all facets of drug-induced behavioural changes, while combining it with additional behavioural parameters is a much more promising approach (193). The current study describes drug-induced changes in seven different motor parameters using a computerised behavioural monitoring system. My study aimed to explore the association of morphine dosage-regimens, biphasic motor behaviour and antinociceptive tolerance over a period of 14 days of repeated morphine administration in rats administered with different doses of morphine. Mapping the relationship of the presented behavioural effects of morphine with its dosing regimen (e.g. starting dose and increment of dosing), will help to reduce these effects by manipulating morphine administration protocols in the future.

In this study, I confirmed that subcutaneous administration of morphine produces locomotor suppression in rats after acute administration (day 1; Fig.13 and Fig. 15), which is in agreement with previous studies showing that even lower doses of morphine can decrease motor behaviour after acute or short-term treatment in rodents (191,193,195,441,456). However, I also showed that repetitive morphine administration (twice a day) resulted in locomotor tolerance (morphine-induced reduction of locomotion) after 5 days, the extent of which depended on the dose administered (partial tolerance at 5 mg/kg b.i.d. and full tolerance at 10 mg/kg b.i.d.). The tolerance profile for the behavioural effect of morphine seemed to manifest in parallel to the manifestation of antinociceptive tolerance, as I recently showed (137). However, one significant distinction between the motor effects of the two doses in my study was that the effects of the lower dose plateaued after 2 days that were non-significantly different from basal levels until 5 days after treatment. The larger dose resulted in the manifestation of the excitatory phase of morphine-induced motor effects shown by the resulted hyperactivity after 5 days of treatment, which plateaued at 10 days after repetitive treatment (Fig. 17). In fact, I was able to show that this excitatory effect of high morphine dosing occurred in parallel to the expression of antinociceptive tolerance (Fig. 21). Similar to the basal antinociceptive effects reported previously (137), basal locomotor activities (locomotor, rotation and rearing activities) were also not affected by repeated morphine dosing over the two week period, which supports previous reports (443). Therefore, morphine showed no residual effects on behaviour or antinociception after repeated dosing over 2 weeks.

Biphasic motor effect of morphine is known for quite a while but has been mainly described in acute and short-term morphine administration protocols with contradicts the role of morphine dosing in this biphasic profile (191,192,193,194,195,196,197). I was able to show a detailed description of the manifestation profile of this excitatory state (i.e. rate of increase and timing

of expression), which is key to understand the underlying mechanisms. Moreover, and perhaps more interesting, is the exposure of the role of morphine dosing regimen in the expression of morphine-induced hyperactivity. This study showed that when the morphine regimen changes after the occurrence of hyperactivity, such that the total morphine amount administered remains the same, but the dosing frequency is reduced (e.g. 10 mg/kg b.i.d. → 20 mg/kg/day). Then, the strong suppressive effect of morphine returns to the pro-hyperactivity levels and results in the manifestation of morphine-induced hypoactivity (Fig. 17). These data also show that the morphine-induced excitatory effect is the result of morphine-induced tolerance on motor behaviour, which can be reversed by an increased dose, similarly to the way antinociceptive tolerance can be reversed by a dose increase (137).

Morphine-induced hyperactivity can be reduced by blocking dopaminergic receptors, but these are not specific to the dopaminergic system (194,198). The higher AUC of behavioural activities of animals treated with morphine over a few days indicates clearly that the locomotion related behavioural effects changed over this time using a specific dose. In the present study, the effects of morphine were dependent on the dose administered or dosage regimen, as the AUCs of locomotor activities were statistically lower (over the total 14 days) with lower doses compared to the higher dose group.

Opioid-induced turning, circling or rotation is mediated by the dopaminergic system (199,200), but not by the μ-opioid receptor (457). Circling or rotating behaviour is thought to be regulated by dopaminergic mechanisms, and circling animals models have been used to assess anti-Parkinson's disease drugs (458). Nevertheless, the rotating or turning behaviour in this study was suppressed in line with general locomotion after acute treatment of morphine and with a similar time kinetic. This agrees with early studies showing a similar suppression of rotation

by morphine in rats (459,460). I also showed that the rotational behaviour is subjected to effects of morphine-induced tolerance, similarly to locomotion and antinociception (Fig. 14), suggesting a strong link of this behaviour to the opioidergic system.

Rearing activity is an exploratory behaviour of rodents and is related to information gathering or cognitive behaviour (205). Little or no rearing in the open-field may indicate motor impairment (206). Therefore not surprising, changes in rearing behaviour are also influenced by benzodiazepine treatment (461), which suggests that other neuronal systems might be more important for this behaviour. Recent studies have related rearing to gamma-aminobutyric (GABA) inhibitory neurotransmission controlled by the $GABA_A$ receptor in the hippocampus (207). Locomotion and rearing are positively correlated and are very reliable factors in terms of exploratory behaviour in untreated animals (192,210). Here, I show that morphine reduces rearing activities due to repetitive treatment with both low and high doses of morphine, in-line with previous studies (462). However, the morphine-induced suppression of rearing was not associated with tolerance, irrespectively of the morphine dose used, the changes in dosing regimen and the length of treatment (Fig. 14). The difference of rearing behaviour compared to the rest of motor behaviours tested (free moving and rotation) could indicate the involvement of additional non-opioidergic systems that affect brain areas that are involved in the motor control of this behaviour, which either alleviates or delays the manifestation of tolerance in rearing.

In summary, the results of this study illustrated that a lower morphine dose reduced the motor behaviour, which was subject to behavioural tolerance after repetitive administration but did not lead to subsequent behavioural hyperexcitation. In contrast, animals treated with a higher morphine dose developed acute motor-suppressive behaviour that quickly desensitised to basal

levels and progressed to an excitatory phase after 10 days, which was in parallel to the development of antinociceptive tolerance. Therefore, morphine dosing plays a crucial role in the manifestation of changed motor behaviour similar to antinociception and motor behavioural tolerance follows a similar pattern to antinociceptive tolerance. In contrast, rearing shows a distinctive resistance to tolerance and dosing changes. My results suggest that morphine dosing determines the expression profile of behavioural effects by morphine and that antinociceptive tolerance is linked to the morphine-induced hyperexcitatory phase of behaviour.

CHAPTER FOUR

Age-dependent antinociception and behavioural inhibition by morphine

Preface to chapter four

I described the role of morphine dosing on antinociceptive tolerance and motor behaviour of 8-week old animals in *Chapter two* and *three*. Clinical studies show that morphine has differential antinociceptive effects in older compared to younger individuals. Since the published results are largely inconclusive, further studies are needed to investigate this observation in more detail. This chapter (*chapter four*) aimed to investigate the effects of morphine on antinociception and motor behaviour in older and younger animals and connect the results to residual morphine levels in post-mortem tissues. In summary, this chapter was designed to increase our understanding of the age-dependent effects of morphine.

4. Age-dependent antinociception and behavioural inhibition by morphine

Abstract

In current clinical practice, morphine is dosed in older patients based on patient-weight, with different calculations for adjustment. However, at present, neither clinical experience nor the literature offers a clear evidence base for the relationship between antinociception, behavioural effects and morphine administration in older patients. In this study, I compared the nociceptive response of 8 and 24-week old rats after subcutaneous administration of morphine *per body weight* and analysed their behaviour using an advanced multi-conditioning system. Residual morphine in all major tissues was determined. I observed prolonged morphine-induced antinociception in older rats compared to younger rats. Moreover, morphine significantly stimulated locomotor and rearing behaviour 180 min after injection, which was significantly higher in the 8-week compared to 24-week old rats. Tissue analysis from animals extracted during the stimulatory phase revealed a significantly higher concentration of residual morphine in the brain of older versus younger animals when standardised on tissue weight. However, this effect was not observed when residual morphine was standardised on protein content. Collectively, the present data suggest that in older rats morphine exhibits higher antinociception and increased behavioural inhibition compared to younger animals. This effect is likely due to a significantly higher accumulation of morphine in the brain of older animals.

Key Words: Morphine; motor behaviour; ageing; antinociception; open-field; locomotion.

4.1. Introduction

The proportion and number of older people are increasing globally with an expected 20% of the total population above 60 years of age by 2050 (463). These individuals experience pain comparatively more than younger people, which affects their daily activities and total quality of life. Noticeably, due to the high prevalence of pain, individuals over 60 years of age are the highest users of analgesics and especially of opioids (405). Therefore, the effective management and safe use of opioids are particularly important for this cohort. Morphine, one of the most frequently used opiates worldwide, is considered a high-risk medication due to its narrow therapeutic index and a plethora of neuropsychological and behavioural effects (406,407,408). Therefore, different calculative dose-adjustments are often clinically used to ensure drug safety but do not always provide effective pain relief (409,410,411,412,413).

Although the prevalence of pain in the ageing population is high, these patients are often not properly assessed regarding their pain relief (464,465,466,467,468), which severely reduces treatment efficacy (469,470,471). A striking lack of data for the ageing population regarding the effects of opioid dosing on patient behaviour is especially evident for patients with cognitive impairment (472,473,474,475). Furthermore, compared to younger patients, aged patients show a higher incidence of adverse events, which are especially associated with long-term opioid treatment (412,476). Therefore, the selection of effective and safe morphine doses in aged individuals is one of the most frequently faced challenges in the clinic. The difficulty to achieve adequate pain relief and at the same time to avert the manifestation of side effects in aged patients has fuelled concerns that factors such as altered drug pharmacokinetics, metabolism or behavioural changes could contribute to this challenge (409,410,411).

Therefore, there is an urgent need to identify a possible correlation between the antinociceptive and behavioural effects of morphine in aged individuals. At present, a limited number of studies suggest a physiological and/or molecular basis for the differences observed in opioid pharmacology when comparing aged and younger individuals (414,415). However, the evident lack of established knowledge regarding the behavioural effects of opioids in aged subjects and the potential differences compared to young individuals hinders the legitimate and appropriate use of opioids in aged individuals.

In this study, I connected the antinociceptive and behavioural activities of morphine with residual morphine concentrations in post-mortem tissues of test animals, to shed some light on the age-dependent pharmacokinetic and behavioural differences of morphine administration.

4.2. Materials and Methods

4.2.1. Materials

Morphine sulphate solution for injection (30 mg/ml) was purchased from Hameln Pharmaceuticals GmbH, Germany. The drug was kept at room temperature in a secured safe in accordance with Australian regulations around schedule 8 drugs. For the low dose group (5 mg/kg), morphine sulphate was diluted to 15 mg/ml with sterile 0.9 % sodium chloride solution immediately prior to subcutaneous (*s.c.*) injection. The injection volumes for both 5 and 10 mg/kg morphine groups were the same according to volume/weight and within each group differed by not more than 8 % when adjusted for individual animal weights. F10SC veterinary disinfectant solution (Health and Hygiene Pty Ltd, Florida Hills, South Africa) was used for cleaning and hygiene purpose as a diluted solution (1:250 in water).

4.2.2. Animals

Male Sprague Dawley (SD) rats (total 20; 4 x n=5) were used in this study. Half of those (n=10) aged for 24 weeks using normal diet (6 % crude fat, Barastoc rodent cubes, Ridley Corporation, Melbourne, Australia) and water, while the other animals (n=10) aged for only 8 weeks on the same diet. During the experimental phase, all animals were single-housed under standard laboratory conditions and kept on an automated 12:12 hours day/night cycle (lights on at 7:00 am). The treated animals were single-housed after administration of morphine and throughout the experiments in order to facilitate behavioural health-monitoring and avert the manifestation of potential morphine-induced behavioural aggression towards cage-mates. Animals were handled for 5-6 days before the experiments were conducted. On the morning of behavioural tests, animals were transported to the testing room in their home case and acclimatised to the test environment for 1 hour. All procedures and animal handling were performed according to the guidelines of the University of Tasmania Animal Ethics Committee (approval no. A13864)

and *The Australian Code for the Care and Use of Animals for Scientific Purposes* (434). Animals of each group (8 and 24 weeks of age) were divided into two subgroups using a completely randomised design as previously described (448). Each group received a different dose of morphine sulphate solution (5 mg/kg or 10 mg/kg) as single *s.c.* injection between the left thigh and the spinal cord. The subcutaneous route of administration was previously shown to be an effective and quick route for morphine administration (Stuart-Harris et al., 2000), causing minimal discomfort to the animals. The two different doses of morphine were selected, based on previous data from our group regarding the connection between morphine loading-dose and antinociception (137). The weight of all animals was recorded prior to the administration of morphine. The BMI of rats was calculated based on the formula: *BMI = body weight (g) / body length^2 (cm^2)* as described previously (477,478). The animals were tested side-by-side by the same operator in the same testing environment, but extensive cleaning and hygiene procedures were undertaken to ensure that the younger animals were not exposed to the scent of the older animals and vice versa. A schematic diagram represents the different time-points before and after morphine treatment of 8 and 24-week old animals (Fig. 23).

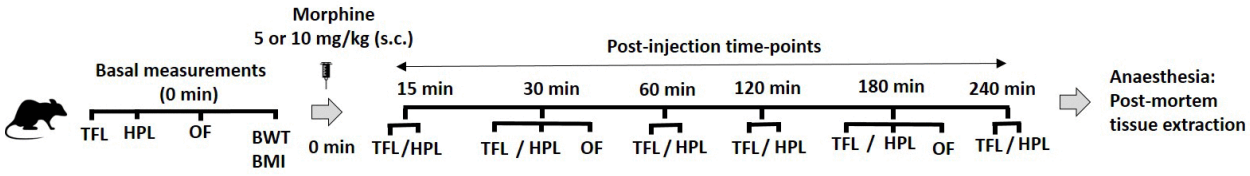


Figure 23. Schematic diagram of the morphine treatment and assessment protocol. TFL: tail-flick latency; HPL: hot-plate latency, OF: open-field test; BWT: body-weight; BMI: body mass index; 0 min: base-line before treatment.

4.2.3. Nociception Measurements

Nociception was determined independently by tail-flick and hot-plate assays performed in random sequence, separated by a 1-minute interval between the two assays, using specialised apparatuses (Ugo Basile, Comerio, Italy). Maximum exposure of the animals to the nociceptive thermal stimuli (cut-off time) was 15 s for the tail-flick and 30 s for the hot-plate assay, as previously described (137,152,437,438). The infrared intensity of the tail-flick photocell was set at 30, whereas the plate temperature of the hot-plate was set at 54 ± 0.5 °C. All rats were tested immediately prior to morphine administration (basal measurement) and at 15, 30, 60, 120, 180 and 240 min post-administration in both assays. The maximum possible effect (MPE) was defined as % MPE = 100 X [(test latency – baseline latency)/(cut-off time – baseline latency)] as previously described (436).

Nociception experiments were conducted blindly and results were recorded by averaging three independent measurements for each time-point with a 1 min difference between measurements to minimise 'handling' effects. No differences in basal nociceptive thresholds were observed in both antinociception assays between 8 and 24-week old rats, which supports two previous studies, that also observed no age-related differences in basal antinociception (479,480). In addition, no differences in antinociceptive latencies over a period of 2 h were detected (data not shown). Similarly, over a period of 2 weeks of repeated testing, basal levels of antinociceptive latencies also remained unchanged (Fig. 9, chapter 3; Suppl. Fig. S4 in (137)), which both indicate that 'testing fatigue' did not affect the experimental results of this study.

4.2.4. Behavioural Measurements

The behavioural testing used six different activity parameters (total distance travelled, rearing time, the ratio of presence in periphery versus centre, clockwise rotation, anti-clockwise rotation and moving time). Behaviour was tested in an open-field arena in a Multi-Conditioning System (MCS) (TSE GmbH, Homburg, Germany) 2 min after nociception-testing at 0 min (pre), 30 and 180 min after administration of morphine over a period of 5 min. Measurements in open field arena over a period of 5 min are commonly used (192,446,450,481) and allowed the concurrent measurements of antinociception in the same group of animals in this study. The MCS platform included an internal noise/light/temperature insulation system and a 3D infrared-beam frame that provided fast and accurate animal movement detection (100Hz), combined with a high-resolution video monitoring and automated movement tracking system. Quantification and visualisation of the MCS data were processed by integrated system software (TSE ActiMot). The open-field arena was thoroughly cleaned between each animal using paper towels soaked in the diluted F10 solution. Background white noise (20 dB) was used during all experiments to cancel out environmental sounds.

4.2.5. Tissue Collection

Immediately after testing antinociception at 240 min post-administration of morphine, animals were anesthetised with 5% (w/v) isoflurane in oxygen at a flow rate of 1 L/min, until loss of consciousness was observed (usually 5-7 min) and the animals were decapitated. Blood was collected from the decapitated body by gravity flow using 15 ml centrifuge tubes (Corning Centristar) and immediately centrifuged at 1500 rpm for 10 min to collect serum. The serum was stored at -80 °C until used in experiments. Serum, spleen, kidney, liver, heart, lungs and brain were collected from decapitated bodies and snap-frozen in liquid nitrogen for the estimation of residual morphine in the animals treated with 5 mg/kg morphine.

4.2.6. Residual Morphine Concentration in post-mortem Tissues

Spleen, kidney, liver, heart, lung and brain tissues were homogenised by an electric homogeniser immersed in ice-cold RIPA buffer. Tissue homogenates were centrifuged (1500 g, 4º C, 10 min) before supernatants were collected for morphine measurements. Residual amounts of morphine were detected in tissue lysates using a morphine ELISA-Kit (#KA0935; Abnova, Taipei, Taiwan) and a Thermo Scientific Multiskan GO microplate spectrophotometer (Thermo Scientific Inc, Waltham, MA, USA). The results were expressed as µg morphine per tissue weight (g) as well as µg morphine per protein content (g) of the tissues extracts. Protein contents of tissue extracts were determined using a commercial protein assay kit (#5000111; Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations.

4.2.7. Statistical Analysis

Data is presented in graphs or tables as mean ± SEM from *n* measurements as stated in the figure legends. Student's t-test was used for comparison of BMIs, one-way ANOVA with Sidak's multiple comparison *post hoc* test was used to compare motor behaviours and body weights, and two-way ANOVA with Sidak's multiple comparison *post hoc* test were used for statistical comparison of antinociception and residual morphine estimation of post-mortem tissues, using GraphPad Prism V6.01 software (GraphPad Software Inc., La Jolla, CA, USA). Repeated measures one- or two-way ANOVA with Sidak's multiple comparison *post hoc* test were conducted to measure the differences between different time points of the same group of animals. However, comparisons between two or more groups of animals were made using non-repeated one- or two-way ANOVA with Sidak's multiple comparisons *post hoc* test. Significance was set at $p < 0.05$.

4.3. Results

4.3.1. Body weight and BMI analysis

Significant differences in body weight between 8 week (253.3 ± 9.6 g) and 24 week old animals (582.1 ± 21.2 g) were observed prior to the initiation of experiments [non-repeated measure one-way ANOVA; $F(5, 26) = 57.77$; $p < 0.0001$] (Table 17). Consistent with increased bodyweight, a significant BMI difference between 8 week (0.83 ± 0.02) and 24 week old animals (1.10 ± 0.05) [unpaired t-test; $t(6) = 5.50$; $p < 0.01$] was detected (Table 17). No weight differences were detected after randomisation between the two different morphine subgroups (5 mg/kg and 10 mg/kg morphine) in the 8 and 24 week old animals (Table 17).

Table 17. Differential body weights and body mass index of 8 week and 24 week old rats

	Body Weight			***BMI***
Age group	***Combined***	***5 mg/kg morphine group***	***10 mg/kg morphine group****	***Combined***
8 week old	253.3±9.6	261.0±16.7	245.7±11.3	0.83±0.02
24 week old	582.1±21.2	617.2±28.3	547.0±24.7	1.10±0.04
p value	<0.0001	<0.0001	<0.0001	<0.01

**No significant differences between 5 mg/kg and 10 mg/kg morphine dose-groups at the same age*

4.3.2. Morphine-induced antinociception in 24 week old rats is prolonged compared to 8 week old rats

No significant differences in basal antinociception were detected for the 8 and 24 week old animals, using both tail-flick and hot-plate assays (Fig. 24). The time-dependent antinociception profiles (herein antinociception "curves") of the 8 and 24 week old animals in response to a single subcutaneous injection of morphine were comparable when measured by tail-flick (Fig. 24 A, C) and hot-plate (Fig. 24 B, D) assays. Antinociception peaked 30 min after morphine injection in both assays, followed by a gradual decrease thereafter. In the tail-

flick assay, antinociception reached the cut-off value in the early post-injection (15-60 min) in both age groups. When the data set of Fig. 24 was analysed using repeated measures two-way ANOVA to identify whether post-morphine administration time and animal age are determinants of morphine-induced antinociception, I observed that both parameters were highly significant [time, $F(6, 96) = 786.7$, $p < 0.0001$; age, $F(3, 16) = 80.90$, $p < 0.0001$]. Significantly higher levels of antinociception were observed in 24 week old rats between 60-240 min post-injection for both morphine doses [e.g. 5 mg/kg: Sidak's $p < 0.0001$ (120 min); 10 mg/kg: Sidak's $p < 0.0001$ (180 min)], which explains the significant differences in AUC values of total antinociception between the two age groups (Fig. 24 A, C; Table 18). In the hot-plate assay, differences in antinociception for both age groups were more pronounced than in the tail-flick assay. These differences became visible for both morphine doses (5 mg/kg and 10 mg/kg) 15 min post-administration [Sidak's $p < 0.0001$] (Fig. 24 B, D). Repeated measure two-way ANOVA underlines that time [$F(6, 96) = 479.9$, $p < 0.0001$] and age [$F(3, 16) = 60.34$, $p < 0.0001$] are the main parameters, with 24 week old animals showing significantly higher antinociception levels after 3 h [Sidak's $p < 0.001$] and 4 h [Sidak's $p < 0.05$] post 10 mg/kg morphine administration (Fig. 24 D). Noticeably, in both assays, total antinociception over 4h by a single morphine injection was significantly higher in the 24 week compared to the 8 week old animals, which is reflected by the Area Under the Curve (AUC) values (Table 18) [non-repeated measure one-way ANOVA; $F(3, 16) = 89.10$; $p < 0.0001$ (tail-flick); non-repeated measure one-way ANOVA; $F(3, 14) = 46.22$; $p < 0.01$ (hot plate)]. The corresponding tail-flick and hot-plate antinociceptive latencies are shown in Fig. 25.

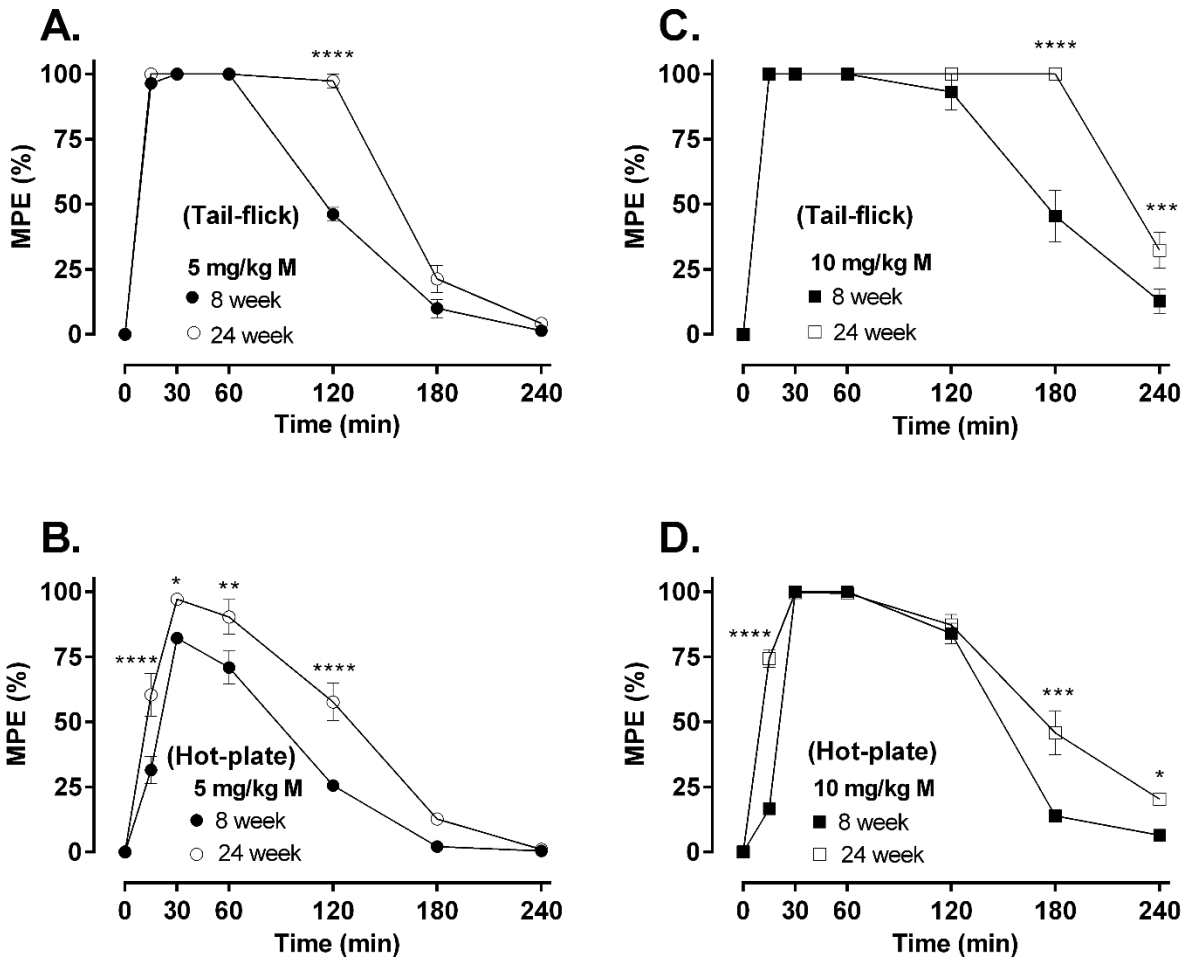


Figure 24. Antinociceptive effect of morphine in 8 and 24 week old rats. Antinociceptive profile of acutely-treated 8 and 24 week old rats with subcutaneous morphine (5 and 10 mg/kg) at pre- and different post-administration times (15-240 min), measured using a tail-flick assay (A, C) and a hot-plate assay (B, D). Antinociceptive curves of morphine 5 mg/kg (A, B) and 10 mg/kg (C, D) are presented as the maximum possible effect (MPE) against pre- and post-administration time-points as described in *Methods*. All data are presented as mean ± SEM, n=5 per group. Statistically significant differences compared against 8 week old animals at the same time-point (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001) using repeated measure two-way ANOVA with Sidak's multiple comparison *post hoc* test. Error bars are present in all graphs but are sometimes too small to be visible.

Table 18. Total antinociception (as area under the curves, AUC) of morphine-treated rats

	Total antinociception of morphine: AUC values (x10^3) from antinociceptive curves			
	Tail-flick assay		***Hot-plate assay***	
Morphine dose	***5mg/kg***	***10mg/kg***	***5mg/kg***	***10mg/kg***
8 week old	11.33 ±0.35	16.45 ±0.59	6.99 ±0.42	13.03 ±0.22
24 week old	15.86 ±0.47	21.22 ±0.21	11.72 ±0.55	16.23 ±1.01
p value	<0.0001	<0.0001	<0.001	<0.01

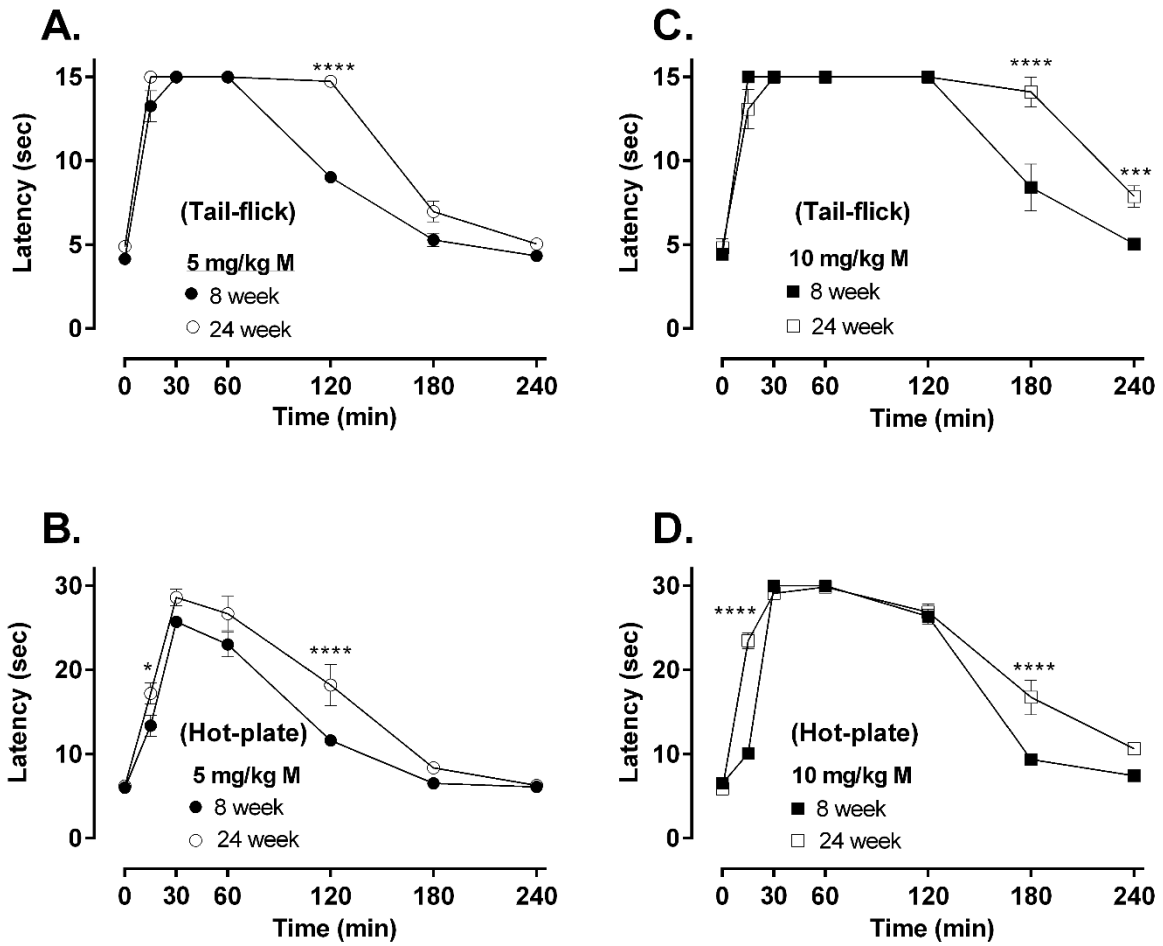


Figure 25. Antinociceptive latencies of morphine in 8 and 24 week old rats. Antinociceptive profiles of 8 and 24 week old rats treated with subcutaneous morphine (5 and 10 mg/kg) pre- and post-administration (15-240 min) was measured using a tail-flick (A, C) and hot-plate assay (B, D). Antinociceptive curves of morphine 5 mg/kg (A, B) and 10 mg/kg (C, D) are presented as latency (in sec) against pre- and post-administration time-points as described in *Experimental design, Materials and Methods*. All data are presented as mean ± SEM, n = 5 per group. Statistically significant differences compared against 8 week old animals for the same time-point were generated using repeated measure one-way ANOVA with Sidak's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). Error bars are present in all graphs but are sometimes too small to be visible.

4.3.3. Morphine-induced behavioural effects post-antinociception of older animals

Despite their differences in weight and BMI (Table 17), 8 week old animals showed higher basal physical activity (distance travelled x rearing(s) occurred) compared to their 24 week old counterparts (Fig. 26; Table 19). Consistently, 30 min after morphine administration, physical activity significantly decreased for both age-groups compared to their basal activity levels [non-

repeated measure one-way ANOVA; F (5, 14) = 9.40; $p < 0.05$ (8 week old); $p < 0.01$ (24 week old)] (Table 19; Fig. 26). After 180 min, activity levels in both 8 and 24 week old animals were indistinguishable from their basal levels. Notably, after 180 min the physical activity of 24 week old animals was significantly lower compared to the 8 week old animals [non-repeated measure one-way ANOVA; F (3, 11) = 14.44; $p < 0.05$] (Table 19). Overall, 24 week old rats showed significantly decreased physical activity compared to 8 week old rats before and after morphine administration (Table 19).

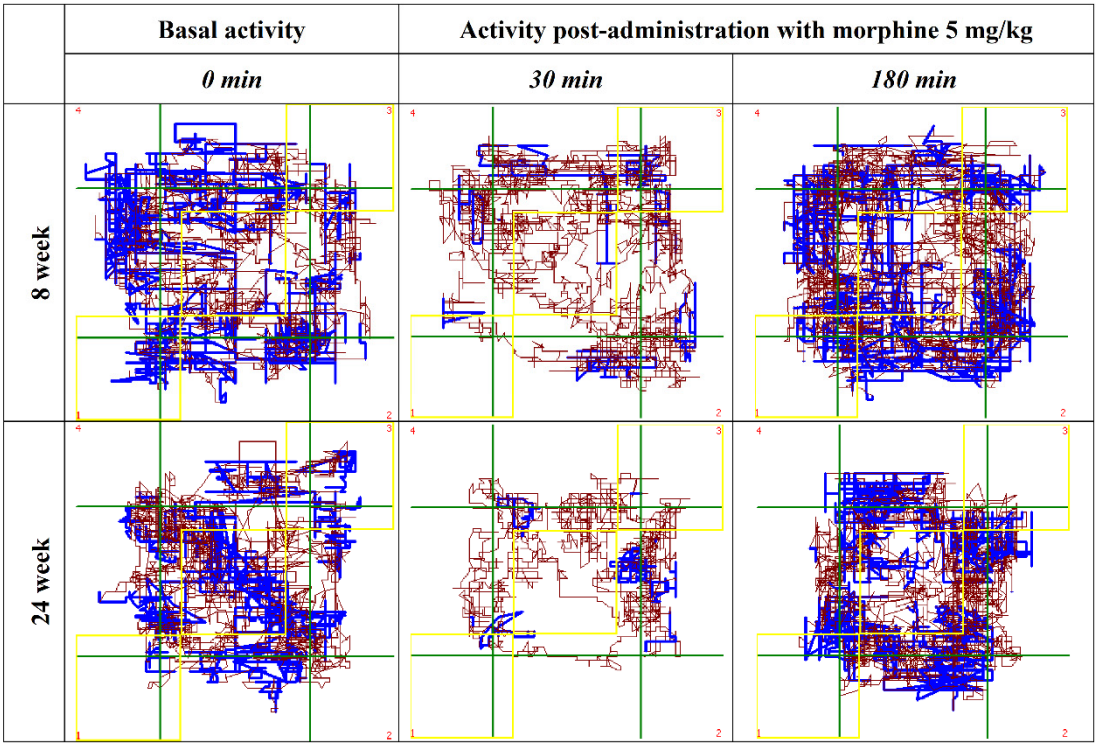


Figure 26. Effects of morphine on total physical activity. Effects of a single dose of morphine (5 mg/kg, s.c.) on the physical activity of 8 and 24 week old rats were recorded in an open-field arena. The pattern of physical activity (A) was acquired using automated video-tracking software that presented the path of movement (brown lines) and rearing (blue lines) during the recorded session. One representative activity pattern for each weight group is shown. Animals were tested at basal (prior to morphine administration), 30 and 180 min post-administration. All data are presented as mean ± SEM, n = 5 per group.

Table 19. Effect of morphine (5 mg/kg) on total physical activity

	Total Physical Activity †		
	0 min	***30 min***	***180 min***
8 week old	3252 ± 832	531.5 ± 126.2	4374 ± 730.3
24 week old	1544 ± 484	70.88 ± 39.37	1706 ± 1058
p value	<0.05	<0.01	<0.05

† *= Distance travelled x rearing incidence (rearing numbers)*

Detailed 3D video-tracking analysis of individual behaviour in 8 and 24 week old animals treated with a single dose of morphine (5 mg/kg) confirmed this effect for three basic behaviours: moving distance, rearing and topology (Fig. 27). The measurement of moving distance (Fig. 27 A) is a marker of locomotion and kinetic behaviour in rodents (191,193,194). A significant reduction of moving distance compared to basal scores was observed in the 8 week old animals at 30 min post-treatment [repeated measure one-way ANOVA; $F(1.2, 4.8) = 47.47$; $p < 0.01$]. These animals also showed a marked increase of moving distance after 180 min, which was significantly higher than their basal values [repeated measure one-way ANOVA; $F(1.2, 4.8) = 47.47$; $p < 0.05$]. Over the same time period, the 24 week old animals showed a very similar response to morphine but at a lower level (Fig. 27 A). Noticeably, moving distances of 8 and 24 week old rats were significantly different for basal [non-repeated measure one-way ANOVA; $F(5, 24) = 32.89$; $p < 0.05$] and 180 post-administration [non-repeated measure one-way ANOVA; $F(5, 24) = 32.89$; $p < 0.05$] time-points (Fig. 27 A). As the second marker of behaviour, rearing activity was assessed, which is a behavioural marker of exploration or anxiety and has been previously linked to brain excitability (205,206,207). In this analysis, 8 week old animals, after a dramatic reduction at 30 min [repeated measure one-

way ANOVA; $F(1.5, 6.2) = 74.68$; $p < 0.01$], showed a marked recovery after 180 min [repeated measure one-way ANOVA; $F(1.5, 6.2) = 74.68$; $p < 0.001$], whereas the 24 week old animals failed to recover from this low level (Fig. 27 B). In contrast, the topology scoring analysis (the distance-travelled ratio for periphery over the centre of arena), which is a behavioural marker for locomotion or anxiety (450,481), differed from the previous two parameters (Fig. 27 C). The 8 week old animals presented an increased topology score after 30 min [repeated measure one-way ANOVA; $F(1.4, 5.7) = 8.79$; $p < 0.05$] compared to the basal score and showed recovery at 180 min time-point [repeated measure one-way ANOVA; $F(1.4, 5.7) = 8.79$; $p < 0.05$] (Fig. 27 C). In contrast, in the 24 week old animals, morphine did not affect the topology behaviour, which remained low throughout the test (Fig. 27 C). A score of nearly 1 for the topology behaviour of 24 week old rats indicated that these animals moved to a similar extend in the periphery or the centre of the arena (Fig. 27 C). Turning behaviour (clockwise and anticlockwise rotation time) and moving time were similar in both groups for basal levels (0 min) and 180 min post-injection of morphine (Fig. 28). However, 24 week old rats showed a significant reduction of turning behaviours compared to 8 week old rats 30 min post-treatment (Fig. 28). The behavioural data of both 8 and 24 week old animals treated with 10 mg/kg morphine showed a similar response to animals treated with 5 mg/kg morphine (Fig. 29). Morphine induced behavioural and antinociceptive effects among the 8 and 24 week old rats are presented in Table 20.

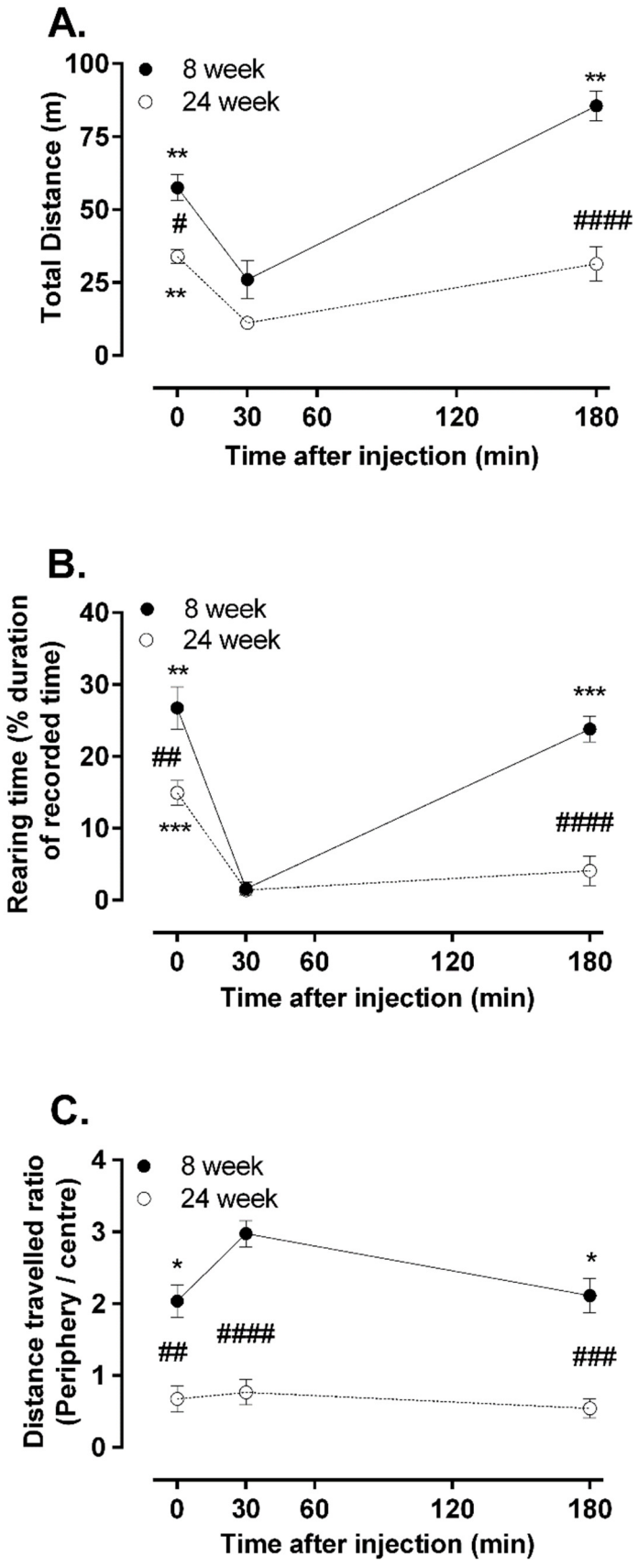


Figure 27. Detailed analysis of morphine-induced behavioural changes. Three distinct animal behaviour (A. total distance, B. rearing time, C. Topology: ratio of distance travelled in periphery vs centre) were recorded after a single-dose of morphine (5 mg/kg, s.c.) in 8 and 24 week old rats and analysed using ActiMot software (TSE Systems). Measurements were taken prior to morphine administration (basal), 30 and 180 min post-administration. The differences between the 30 min time point and basal or the 180 min time point were significantly different (*$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$, using repeated measure one-way ANOVA with Sidak's multiple comparison *post hoc* test) or the significant differences against 8 week old animals at each time points ($^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, using non-repeated measure one-way ANOVA with Sidak's multiple comparison *post hoc* test). All data are presented as mean ± SEM, n = 5 per group. Error bars are present in all graphs but are sometimes too small to be visible.

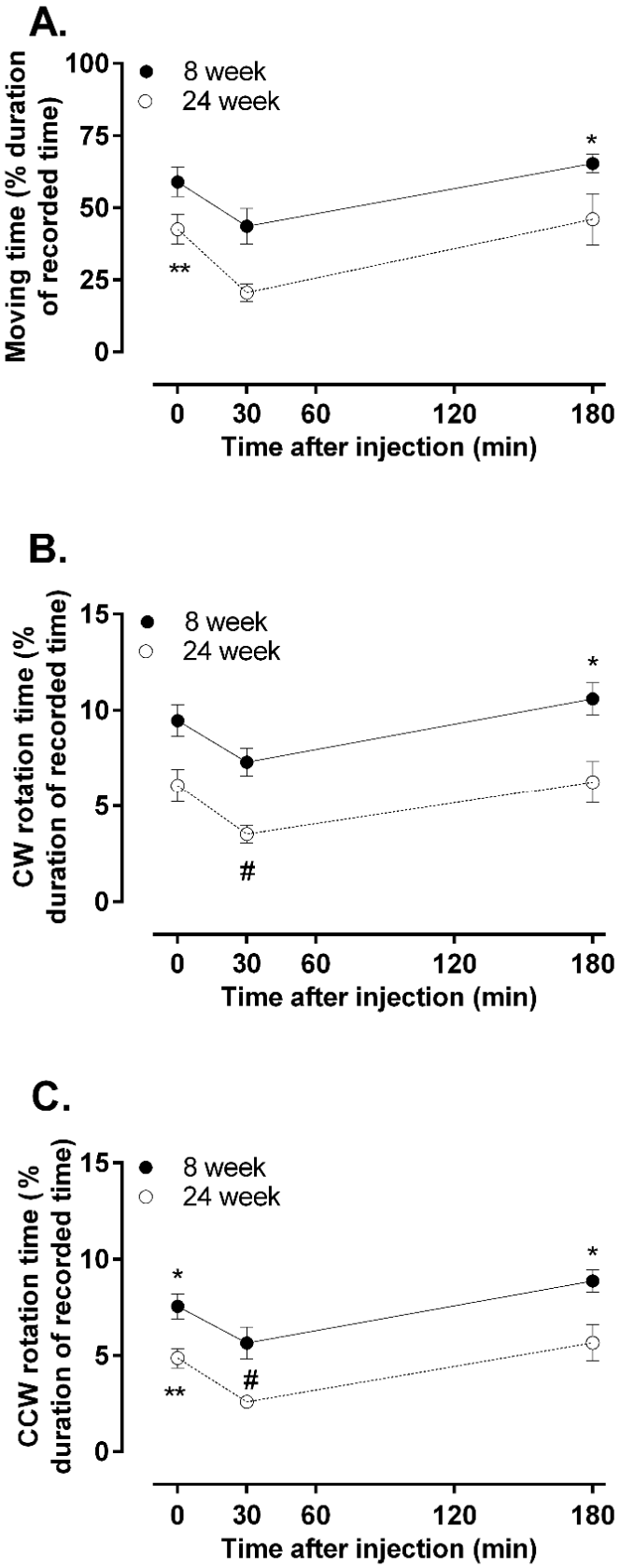


Figure 28. Detailed analysis of low-dose morphine-induced behavioural changes. Three distinct animal behaviour (A. Moving time, B. Clockwise (CW) rotation time, C. Counter-clockwise (CCW) rotation time) were recorded after a single-dose of morphine (5 mg/kg, s.c.) in 8 and 24 week old rats and analysed using ActiMot software (TSE Systems). Measurements were taken prior to drug administration (basal), 30 and 180 min post-administration, as described in *Experimental design, Materials and Methods*. Differences between the 30 min time point and the basal or 180 min time points were significantly different (*$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$, using repeated measure one-way ANOVA with Sidak's multiple comparison *post hoc* test). Likewise, significant differences against 8 week old animals were observed for some time points ($^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, using non-repeated measure one-way ANOVA with Sidak's multiple comparison *post hoc* test). All data are presented as mean ± SEM, n = 5 per group. Error bars are present in all graphs but are sometimes too small to be visible.

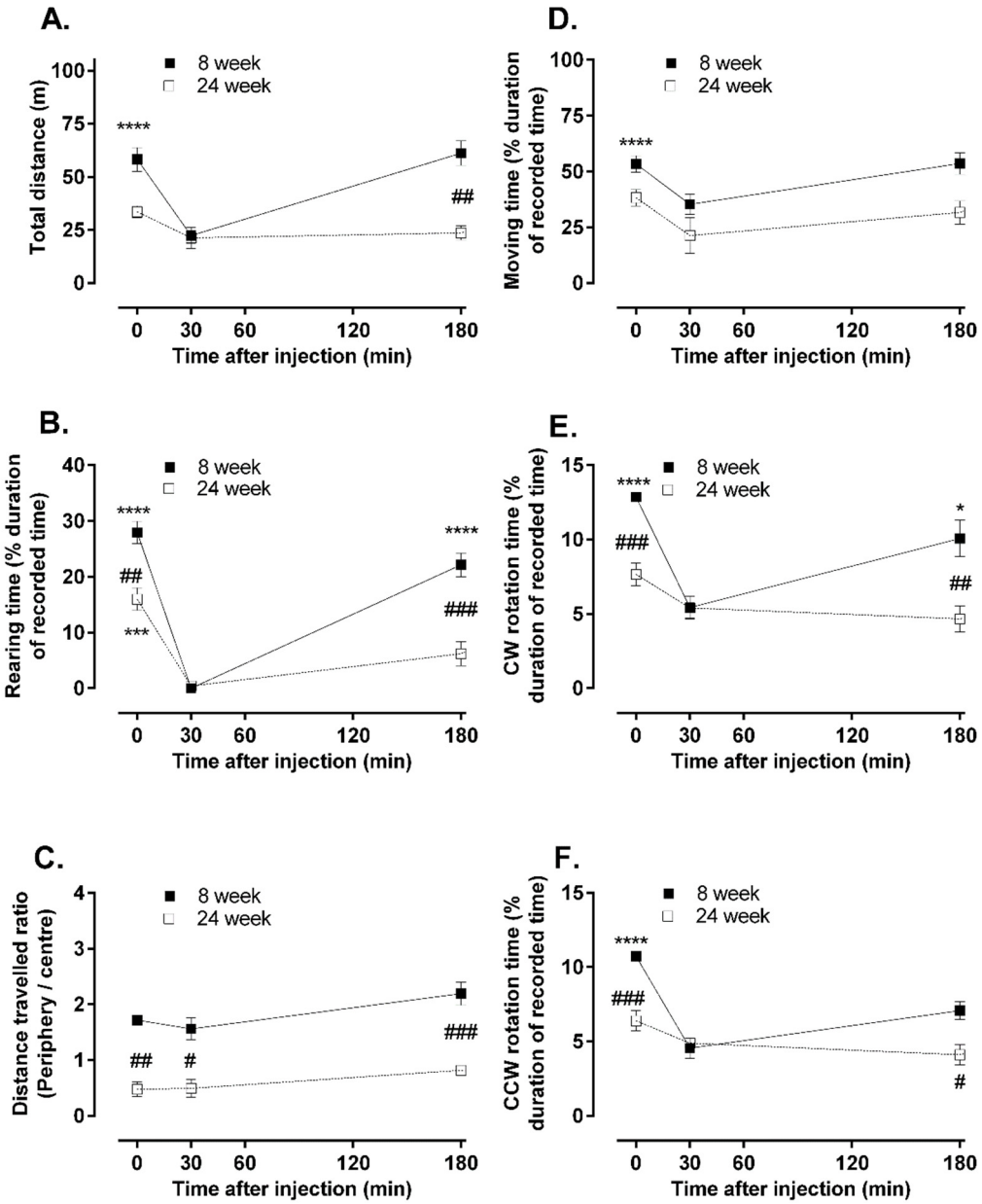


Figure 29. Detailed analysis of high dose morphine-induced behavioural changes. Six distinct animal behaviour (A. Moving time, B. distance, C. rearing time, D. clockwise (CW) rotation time, E. Counter-clockwise (CCW) rotation time, and F. topology: ratio of distance travelled in periphery vs centre) were recorded after a single-dose of morphine (10 mg/kg, s.c.) in 8 and 24 week old rats and were analysed using ActiMot software (TSE Systems). Measurements were taken prior to drug administration (basal), 30 and 180 min post-administration, as described in *Experimental design, Materials and Methods*. Differences between the 30 min time point and the basal or 180 min time points were significantly different (*$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$, using repeated measure one-way ANOVA with Sidak's multiple comparison *post hoc* test). Likewise, significant differences against 8 week old animals were observed for some time points ($^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, using non-repeated measure one-way ANOVA with Sidak's multiple comparison *post hoc* test). All data are presented as mean ± SEM, n = 5 per group. Error bars are present in all graphs but are sometimes too small to be visible.

Table 20. Overall differences of morphine-induced antinociceptive and behavioural effects in 24 week old compared to 8 week old rats.

	Morphine induced behavioural effects					
Tests	***Anti-nociception***	***Speed travelled***	***Distance travelled***	***Rearing***	***Topology[#]***	***Rotation***
Effects for 24 vs 8-week old animals	Prolonged	Similar	Prolonged inhibition	Prolonged inhibition	No effect	Prolonged inhibition

[#]Topology: the distance-travelled ratio for periphery over the centre of the arena

4.3.4. Increased residual morphine in the brain of older rats

To understand whether the observed stimulatory effects of morphine post-antinociception were related to tissue concentrations, residual tissue morphine levels were quantified at the end of the behavioural stimulatory phase (Fig. 30). The analysis of morphine per tissue-weight showed significant differences between 24 and 8-week old animals for several tissues (spleen, heart and lung). Using non-repeated measure two-way ANOVA, age [$F (1, 28) = 45.82$; $p < 0.0001$] and tissue type [$F (6, 28) = 28.44$; $p < 0.0001$] were identified as the main effectors. Most importantly, the analysis revealed that significantly higher morphine concentrations were detected in the brain [Sidak's $p < 0.05$] of 24-week old animals compared to the 8-week old group (Fig. 30 A). Since the weight gain in 24-week old animals could be attributed to increased muscle mass or increased amount of fat deposition in the tested tissues, I also standardised residual morphine levels on tissue protein content to compensate for the fact that morphine can accumulate in fatty environments. Using non-repeated measure two-way ANOVA, significant effects of age [$F (1, 28) = 49.26$, $p < 0.0001$] and tissue type [$F (6, 28) = 16.86$, $p < 0.0001$] with morphine tissue concentrations were observed. Also, significant differences were

observed in serum [Sidak's $p < 0.0001$], spleen [Sidak's $p < 0.0001$] and kidney [Sidak's $p < 0.05$] compared to a standardisation against tissue weight (Fig. 30 B). Tissues with previously significant age-dependent differences in residual morphine between such as heart, lung and brain, largely lost that significance when the morphine levels were standardised on tissue protein concentrations. Finally, I displayed the ratio of morphine present 24 week versus 8-week old rats either as *morphine per tissue weight* or *morphine per tissue protein* content to explore if tissue fat content could affect the results (Fig. 30 C). No obvious differences were observed in most tissues except the heart and brain. The corresponding amounts of morphine shown in Fig. 30 are also represented in Table 21 to illustrate the difference between 8 and 24-week old animals.

Table 21. Residual Morphine content in post-mortem tissues after 4 hours of injections of 5 mg/kg morphine

Residual morphine (μg) (± SEM)						
Tissue	***8 week***	***24 week***	***p value***	***8 week***	***24 week***	***p value***
	per tissue weight(g)	***per tissue weight (g)***		***per protein content (g)***	***per protein content (g)***	
Serum	4.47 ± 1.72	23.32 ± 1.61	> 0.05	0.49 ± 0.12	3.39 ± 0.34	< 0.0001
Spleen	22.45 ± 2.93	68.41 ± 7.05	< 0.001	0.89 ± 0.14	4.63 ± 0.35	< 0.0001
Kidney	58.66 ± 5.65	56.14 ± 5.95	> 0.05	2.42 ± 0.53	4.23 ± 0.15	< 0.05
Liver	40.12 ± 6.46	44.52 ± 1.01	> 0.05	3.25 ± 0.37	2.81 ± 0.71	> 0.05
Heart	1.62 ± 0.31	20.49 ± 2.31	< 0.0001	0.43 ± 0.31	1.20 ± 0.62	> 0.05
Lungs	17.38 ± 3.61	63.22 ± 14.8	< 0.001	0.96 ± 0.32	2.28 ± 0.54	> 0.05
Brain	1.21 ± 0.49	7.47 ± 0.82	< 0.05	0.25 ± 0.11	0.41 ± 0.18	> 0.05

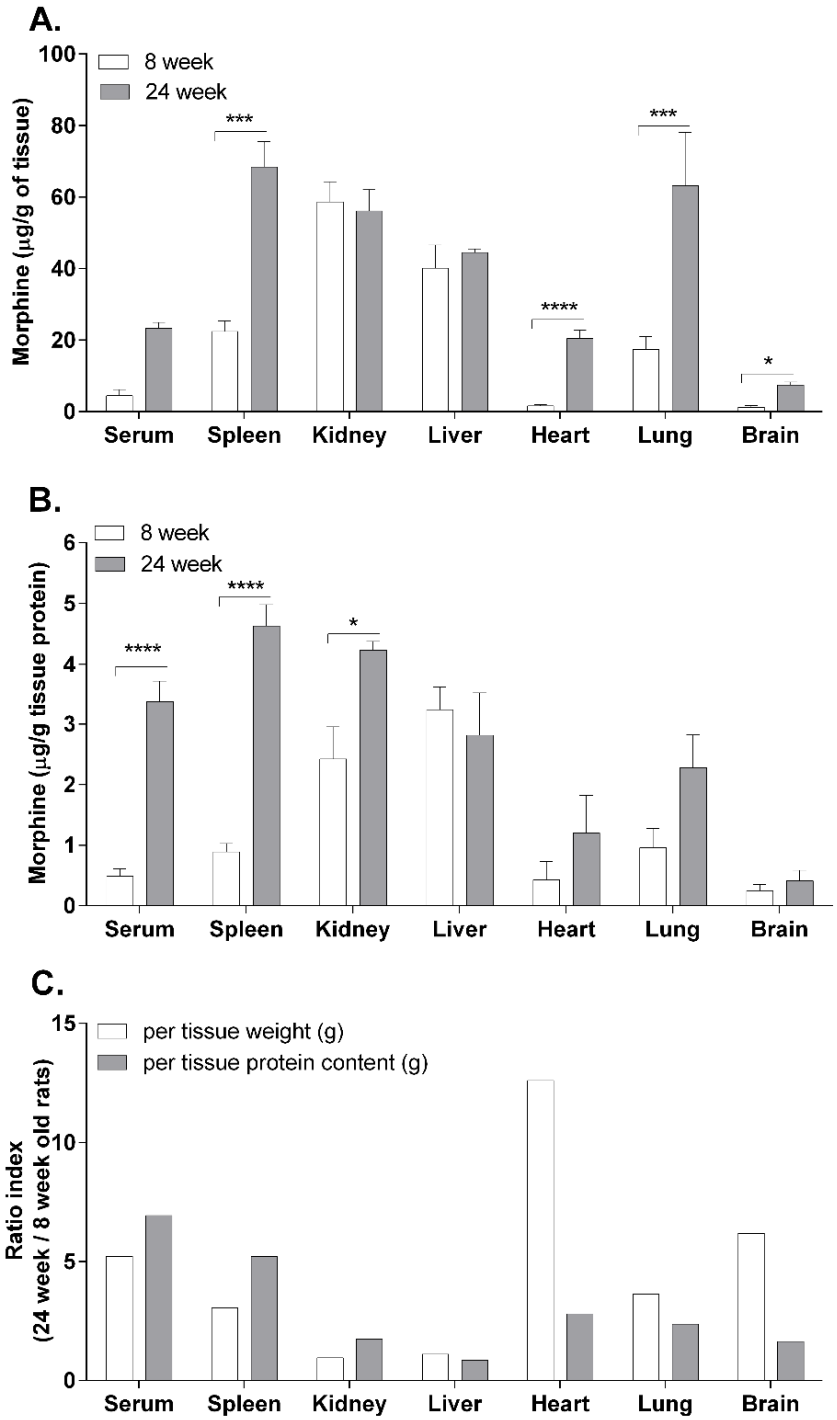


Figure 30. Residual morphine content in post-mortem tissues. Levels of residual morphine were detected in tissues from 8 and 24 week old rats treated with a single-dose of morphine 5 mg/kg after 240 min post-administration, as described in Methods. Amounts are expressed as µg of morphine per total weight (g) of tested tissues (A) and as µg of morphine per protein content (g) in the tested tissues (B). Residual morphine in specific tissues was also displayed as morphine *per tissue weight* versus morphine *per tissue protein content* (C). Significant differences against 8 week old animals are listed for each tissue (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), using non-repeated measure two-way ANOVA with Sidak's multiple-comparison *post hoc* test. All data are presented as mean ± SEM, n = 5 per group.

4.4. Discussion

Current clinical practice for administering morphine to elderly patients involves appropriate dose adjustments based on the patient's weight to provide appropriate analgesia and to minimise adverse effects (482,483,484). To achieve adequate pain-relief in elderly individuals, a lower morphine dose can be necessary due to age-related changes in pharmacodynamics or pharmacokinetics (482,484). This study aimed to investigate the possible correlation between antinociception and behavioural changes, which I compared in older and younger animals after a single morphine dose standardised to body weight. Initially, I compared the antinociception profiles of older and younger animals before and after treatment with two different morphine doses. Secondly, compared the behavioural responses of older and younger morphine-treated animals were recorded. In the final component of the study, residual morphine levels in different tissues of older and younger animals were compared.

At present, evidence for differences in age-dependent morphine-induced antinociception in pre-clinical animal models is inconclusive. In addition, there is also a paucity of studies that describe age-dependent behavioural data as a surrogate marker of morphine-induced side-effects in rats. In this study, I showed that 24 week old rats exhibited morphine-induced analgesia over a longer time period compared to 8 week old rats. This result supports clinical data that described a longer duration of analgesia in elderly patients (416). In addition, in two clinical studies, younger patients required higher doses of opioids to provide similar levels of pain-relief compared to older patients (485,486).

However, at the same time, my results appear to contradict several pre-clinical studies, where older animals either showed lower antinociceptive responses compared to younger animals receiving a weight-adjusted morphine dose (417,418,419,420,421) or no difference at all (422).

These differences are likely the consequence of differences in animal species, age of animals, testing methods and experimental conditions between these and my study. All these studies used different strains of mice or rats and tested antinociception under significantly different experimental conditions compared to the present study (417,418,419,420,421,422).In contrast to my study, 3-6 month versus 24 month old animals was compared in one study (Van Crugten *et al.* 1997), while other studies used multiple age groups between 2 and 31 months, which makes direct comparisons nearly impossible (417,418,419,420,421).

Morphine-induced behavioural effects are not commonly studied in combination with its antinociceptive effects. In the present study, older animals showed significantly lower levels of physical activity compared to the younger animals. While 3 hours after morphine injection this activity in the younger animals returned to basal levels, this effect was not observed in the older animals. More pronounced changes were observed in the topology of locomotion (ratio of distance travelled between periphery/centre), which is an indicator of an anxiety as well as an exploratory activity (450,481). The decreased topology of locomotion in older animals in the present study suggests that older animals were less anxious compared to their younger counterparts, as younger animals travelled more in the periphery of the open field arena. However, at the same time, along with decreased physical activity and distance, it indicates older animals were less explorative than younger animals. The reduced physical activity of older animals compared to younger animals could be explained with the observation that older animals generally display reduced levels of natural activity. Therefore this might not necessarily reflect anxiety-like behaviour. A previous study reported no differences in locomotor activity between older (24-27 months) and younger (3-6 months) mice during a 2 hour observation period after the administration of 10 mg/kg morphine (421). Since this study used much older animals of a different species compared to the present study, differential

locomotor activities are not necessarily unexpected. In addition, this study only described horizontal locomotor activities (421), which is insufficient to reflect the total motor behavioural effects of morphine (193). In contrast, the present study describes the behavioural effects of older and younger rats using six different types of motor behaviour that are related to horizontal locomotion, rearing and turning.

Morphine-induced detailed locomotor activities were measured as behavioural effects of the animals that allowed me concomitant measurements of nociception and behaviour. Behavioural measurements like addiction, anxiety and mood disorders need new groups of animals, additional drug-dosing regimen and different protocols for habituation, treatment and measurements (170,487,488,489). Most importantly, those testing schedules would not allow a concomitant antinociception measurement. Therefore, my study focussed specifically on detailed locomotor activities to measure different opioid-induced behavioural changes. Acute treatment of morphine excites dopamine neurons (DA) to release the neurotransmitter dopamine in the ventral-trigeminal area (VTA) and nucleus accumbens (NAc) (490,491,492,493). The nigrostriatal pathway transmits dopamine from the substantia nigra pars compacta (SNc) to the dorsal striatum (494). Dorsal striatum coordinates motor functions by receiving neurotransmission from different parts of the brain. DA neurons also synapse into GABAergic neurons, which releases the neurotransmitter GABA (494). Studies also show that morphine-induced MOP receptor activation reduces local GABAergic transmission, which is responsible for the stimulation of dopamine release DA of VTA (493,495,496). The activation of dopaminergic neurons in both VTA and NAc regions by acute or repeated morphine can induce the firing of DA and dopamine release, which also depends on the activation of NMDA receptors (493,497). The increased dopamine in the VTA and NAc regions controls locomotor activities (reduced or biphasic locomotion), which also depends on the frequency of

administration of opioids (491,493,498). The inhibitory or stimulatory effects of morphine on locomotor activities are probably controlled by the neurotransmission of dopamine and GABA in the brain. In agreement with previous reports, this study confirmed that morphine produces a biphasic behavioural profile for most motor behaviours (191,193,441,447), where an initial suppressive phase is followed by a phase of overcompensation 180 min after drug administration. However, my data clearly show that this biphasic effect is absent for most morphine-induced behaviours in 24-week old rats, where the inhibitory phase prevails over the entire observation period up to 180 minutes post-administration. It is important to note that the suppression of behavioural activities observed the older animals in this study could be simply interpreted as the result of their higher body weights. Although the higher body weight of the older animals in the present study is likely a consequence of increased muscle and/or fat content, the exact estimation of these parameters was beyond the scope of this study design and will have to analyse in more detail in future studies.

The present study also showed that the morphine-induced biphasic behaviour was inversely correlated with the analgesic profile of morphine, where the time points for highest antinociception aligned with the time points for lowest behavioural activity. Similarly, the over-active behaviour coincided with the time-period when the antinociception effect of morphine had dissipated. However, in contrast to this pre-clinical study, the analgesic and behavioural side-effects of morphine appear to be poorly correlated in the clinic (190) and clinical studies furthermore appear to contradict each other. While some studies reported drug-induced drowsiness or lethargy (452,499), others described euphoria or anti-depressant activity (453,500). Therefore, the translation of the preclinical results of this study to the clinic has to be approached with significant caution.

When I analysed tissue morphine levels 4 hours after injection, increased residual morphine was detected in some tissues of older animals. However, this effect was dependent on the method of standardisation. While increased morphine levels were detected in the spleen, heart, lung, and brain after standardisation to tissue weight, I only observed significantly increased levels of morphine in serum, spleen and kidney after standardisation to tissue protein content. However, two previous reports failed to observe age-dependent differences in brain morphine (421,422). The reason for this discrepancy is not clear, but could be the consequence of different rat strains (hooded Wistar rats, Van Crugten et al.), animal species (ICR mice, Hoskins et al.), significantly shorter test periods (only up to 150 min post-injection, Van Crugten et al.), different methods of tissue analysis (HPLC analysis, Van Crugten et al.; radioactivity measurement, Hoskins et al.) as well as different methods of statistical analysis (unpaired t-test, both studies) (421,422). In the present study, residual morphine was not determined for the higher morphine dose of 10 mg/kg, as both the 5 and 10 mg/kg doses showed similar maximum antinociception and behavioural inhibition at 30 min post-injections with a prolonged duration of these effects. Since both morphine doses showed similar differences between the 8 and 24 week old animals, residual morphine concentrations in tissues of the higher dose group were not measured.

Due to the widespread experimental heterogeneity of pre-clinical studies (417,420,501,502), the largely inconsistent data come as no surprise. One uncertainty generally associated with pre-clinical models is the definition of animal age and its translation to human age (503,504). Most studies defined animals aged between 2 and 12 months as ‘young’ and animals aged 12 to 30 months as aged. However, one important age-dependent parameter that cannot be ignored in this context is the increased body weight of older animals. Rats in captivity accumulate significant body weight, which is thought to be the consequence of age-dependent increases in

lipid content (505,506). Consequently, pre-clinical rat models confront us with the challenge that drug-dependent effects could be modulated simultaneously by both age-dependent and body weight-dependent mechanisms, which are intrinsically linked under standard animal husbandry conditions and have to be seen as a limitation of this approach. In this context, I acknowledge that the present study did not explore the age-dependent pharmacokinetics of morphine. Apart from time-resolved plasma levels, future studies will have to test, for example, if the fat pads in the animals could act as a reservoir for residual morphine, which could produce a delayed drug-release over time.

Although the mechanism behind this prolonged antinociceptive and behavioural inhibition of morphine in older rats is not clear, it is reasonable to hypothesise that this effect is related to the significantly higher accumulation of morphine in the brain of older animals. It has to be noted that the average weight difference between 24 and 8-week old rats was only 2.3-fold, while an up to 6.2-fold higher residual morphine concentration was detected. This could suggest the presence of predominantly age-related pharmacokinetic and pharmacodynamic differences in this study, which are not predominantly dependent on body weight. However, this interpretation is not necessarily supported by clinical data, where a lower dose of morphine was required in older subjects to maintain similar levels of pain-relief compared to younger patients (482,484). In one of these studies, elderly patients showed significantly lower body weight than younger patients (484). These discrepancies highlight the likelihood that the physiological responses to morphine are governed by multiple age-dependent parameters that most certainly include patient weight, fat content and ADME parameters.

Apart from the differences in body weight, body composition and drug-drug interactions (423,424), altered ADME parameters represent a potential cause for different responses to morphine treatment in aged patients. Older patients with impaired cytochrome P450 (CYP) enzyme activity and/or renal impairment, are known to be susceptible to opioid toxicity (424,425,426,427,428). Similarly, G protein-coupled receptor kinases (GRK2, GRK6) and β-arrestin expression decrease in an age-dependent manner (507). Repeated opioid exposure leads to inactivation of opioid receptors by G protein-coupled receptor kinases (GRKs) by phosphorylation and subsequent β-arrestin binding, which reduces the efficacy of opioids (508,509,510). Therefore, reduced β-arrestin binding and GRK activity in older animals could increase antinociceptive effects compared to younger animals at the same morphine dose. Detection of CYP, GRKs and β-arrestin expression was beyond the scope of this study and thus, age-dependent changes in these proteins cannot be ruled out as the underlying reason for the observed effects and will have to be investigated in future studies. My study did not explore any possible differences of antinociceptive or behavioural tolerance of morphine between younger and older animals, as the animals were culled to analyse the residual morphine content present in different post-mortem tissues after acute treatment. Since any possible differences are highly relevant for the clinical setting with an increasingly older population that uses opioids, future studies should explore age-related differences in the development of antinociceptive tolerance after chronic opioid treatment.

In summary, my study identified differences in the antinociceptive and behavioural effects of morphine between older and younger rats. Prolonged morphine-induced antinociception and sustained inhibition of motor behaviour in older compared to younger animals were observed for weight-adjusted morphine dose, which was likely a consequence of higher residual morphine in the brain of older rats. My results will inform future studies that aim to investigate

age-dependent behavioural effects of morphine in relation to its analgesic effects and it will be interesting to see if similar effects can be observed for related anaesthetics and narcotics. The findings of the present study support previous studies that infer that older patients should experience sufficient pain-relief with a lower dose of morphine compared to younger patients (482,484,511). Although my study cannot be used to explain the clinically observed differences in the response to morphine treatment between older and younger patients, my observations, together with previous results can aide future studies to thoroughly investigate the pharmacokinetics of morphine and other analgesics specific to the elderly population.

CHAPTER FIVE

Opioid receptor-dependent modulation of insulin-release in pancreatic beta-cells

Preface to chapter five

The previous chapters (*chapters two, three and four*) described different aspects of opioid use such as antinociception, antinociceptive tolerance and behaviour. Pre-clinical studies demonstrated increased MOP receptor expression in the liver and muscles of diabetic rats (390,512,513,514), but the actual consequences of this altered expression on the diabetic phenotype are obscure. To investigate a possible influence of opioids for the management of diabetes, extensive studies are required to understand the relationships between the opioidergic and the pancreatic metabolic system. Currently, a major gap exists in the literature on the effects of the three major opioid receptors (MOP, DOP and KOP receptors) on insulin homeostasisThis chapter (*chapter five*) aimed to understand the individual roles of the classical opioid receptors on insulin secretion.

Chapter 5. Opioid receptor-dependent modulation of insulin-release in pancreatic beta-cells

Abstract

In this study, I aimed to look at the effects of opioid-receptor selective agonists and antagonists on insulin secretion in the pancreatic β-cell line RIN-5F. Cells were treated for 24 hours with 1μM of selective agonists (DAMGO for MOP, DPDPE for DOP, U50488 for KOP) in the presence or absence of 10μM of selective antagonists (CTOP for MOP, naltrindole for DOP, norBNI for KOP). An enzyme-based immunoassay was used to detect the amount of insulin in the supernatant, using standard curve generated from known concentrations of rat insulin. A trypan blue viability assay was performed to assess the toxicity of each treatment and the secreted insulin was expressed as per 10^3 viable cells. Treatment with the DAMGO or DPDPE caused an increase in secreted insulin by 94.2% and 76.3% respectively, compared to the non-treated controls. Co-treatment of DAMGO and CTOP was able to cancel out the agonists' effect. However, CTOP itself was able to increase insulin secretion by 72% when compared to control. These results suggest that opioid-induced insulin secretion may be based on G-protein independent mechanisms. In addition, none of the KOP-receptor selective ligands was able to significantly affect insulin secretion compared to control, whereas naltrindole was highly toxic to pancreatic β-cells since it induced maximum cell death. Collectively, these findings suggest that MOP and DOP receptor-binding opioids might be more relevant in increasing insulin secretion from pancreatic β-cells than KOP receptor ligands.

Keywords: Opioid; insulin; morphine; pancreas; diabetes; glucose; hyperglycemia

5.1. Introduction

Diabetes mellitus is a metabolic disease that is characterised by a disturbance in glucose and insulin homeostasis. Insulin is excreted centrally by the nervous system and locally by the pancreatic β-cells in response to plasma glucose levels and other endocrine signals (515). Diabetes is clinically divided in two types: Type 1 (T1DM) results from failure of the pancreas' ability to produce enough insulin to regulate the body's metabolism, mainly due to an autoimmune degeneration of pancreatic β-cells (516). Type 2 (T2DM) manifests as an initial phase of cell desensitisation to prolong insulin secretion (called insulin resistance) and a second phase of declined insulin secretion; manifestation which is linked to excessive plasma glucose levels and increased body weight (355,356). Based on this classification, one can conclude that glucose and insulin are key players in diabetes mellitus since their dysregulation, along with other endocrine substances such as glucagon released from pancreatic alpha-cells (517), play a catalytical role in the development and the progression of diabetes.

Insulin secretion, as shown by Ammala et al, is triggered by glucose treatment in the same manner as the repetitive electrical stimulations, through a calcium-dependent exocytosis (518). However, since the glucose effect on pancreatic cells follows a step-wise format, insulin secretion follows a characteristic biphasic time-dependent format, with the first phase of secretion within 10–15 mins, followed by a slower second phase (519). This biphasic insulin secretion is important in understanding opioid-induced insulin secretion. Since impaired insulin secretion from pancreatic β-cells is a major factor in the pathology of both types of diabetes mellitus, it is only rational that many studies have focused on investigating the effect of different drugs on this process. Although there are a number of regulators of insulin secretion, such as plasma glucose levels, circulating hormones, paracrine and autocrine signals, the main player in the regulation of β-cell secretion is insulin itself, since it has been reviewed in

maintenance and regulation of β-cell function (515,520), another reason why insulin secretion is such a critical factor in the pathophysiology of diabetes.

Patients with diabetes mellitus may develop different types of painful neuropathies. About 30% of diabetic patients experience neuropathic pain due to nerve injury induced by diabetes and about 20% of patients will develop chronic pain at some point during their life (361,362). Although opioids like morphine and fentanyl are considered ideal medications for medium-severe pain, their efficacy in diabetic neuropathy is as limited as in other types of neuropathic pain (363). Nevertheless, the combination of opioids with non-opioid drugs that are effective in neuropathic pain (such as α_2 adrenoceptor agonists and serotonin/noradrenaline reuptake inhibitors) has proven to offer certain therapeutic advantages (521), while it is being used in cases of diabetic neuropathies where first-line treatment with anticonvulsants or antidepressants was ineffective (363,522,523).

Opium has been used for thousands of years due to its various therapeutic and recreational properties (i.e. analgesia, euphoria, drowsiness). However, it was only after the discovery of the three classical opioid receptor types (mu, MOP; delta, DOP; kappa, KOP) (524) and their endogenous ligands (endorphins, enkephalins and dynorphins respectively) that I increased our understanding of the opioid system and made opioid drugs one of the few classes of compounds that have been studied for so long (525). Opioid receptors are found in a wide variety of tissues, including the hypothalamic-pituitary system, while their role and effect in endocrine regulation has been well-studied during the last decade (526,527). All opioid receptors (MOP and DOP primarily, KOP in less extent) have been shown to be expressed in the pancreas and liver, while endogenous opioid peptides and their receptors have been shown to be present in pancreatic α- and β-cells (528,529,530).

Nevertheless, the first published work on the identification of endogenous opioids in the pancreas (531) gave rise to studies that focused on the ambiguous mechanisms involved in the endogenous opioid-mediated glucose and insulin homeostasis (387,392,532). Green et al first showed that morphine and met-enkephalin were able to stimulate glucose-induced insulin release in isolated pancreatic isles (392), an effect that was observed to develop rapidly, representing the first phase of pancreatic insulin release and which could be blocked by naloxone pre-treatment and therefore, showing an opioid-receptor mediated effect. A number of studies that followed confirmed the results of Green et al either directly (395,396,397) or indirectly by showing a decrease in insulin secretion by administration of naloxone in healthy humans (398) and which highlighted the role of endogenous opioids on pancreatic function.

Blockage of endogenous opioids by naloxone (an opioid antagonist) was reported to improve hypoglycaemia in type-1 DM patients (388). The study indicates that opioids can reduce blood glucose levels in human subjects (388), which is in line with the observation that opioids can stimulate insulin secretion in a pancreatic β-cell line (390). However, although more than 30 years have passed since the first studies on the endocrine effects of opioids, there hasn't been a sufficient progress towards a clear understanding about the role of opioid receptors and endogenous opioids in insulin secretion, as reflected by the small volume of published work on this area of research compared to the length of period studied. Part of the reason for this delay in progress is the considerable controversy that exists in the literature regarding the contribution of the three classical opioid receptors and their ligands to the mechanisms and pathways mediating glucose-induced insulin secretion. A number of studies have shown a stimulatory effect of opioid agonists (392,393,394,395,396,397,398), while others have shown the opposite inhibitory effect (399,400,401,402,403,404).

A major hypothesis for this inconsistency is the different methodologies employed and the different models used in detecting insulin levels (515,533). The insulin signalling system is auto-regulated through different homeostatic mechanisms that involve glucose and glucagon secretion (among others). It is, therefore, most likely that observations of insulin secretion using high-complexity models (e.g. animal studies, perfused organs, mixed-cells tissues, etc.) would differ from observations performed in primary β-cell lines. Many *in vitro* studies that I discuss later in this chapter, have used isolated pancreatic islets to study glucose-induced insulin secretion, although these isolates include different types of cells (α-cells, β-cells, γ-cells) that produce different signals under certain conditions (e.g. glucagon, insulin, neuropeptide Y, amyloid peptide, ATP, GABA and other peptides) which can contribute differentially to the resulted insulin secretion (515). This heterogeneity may qualify studies using β-cells (the main cells secreting insulin in the pancreas) as more accurate in terms of drug-screening on insulin secretion (534).

Nevertheless, a major gap in the literature is the investigation of the individual role of the three classical opioid receptors in insulin secretion, using receptor-selective agonists and antagonists. Interestingly, no one looked at the effects of selective activation of MOP, DOP and KOP receptors in glucose-mediated insulin release in a pancreatic β-cell line. A detailed investigation into the role of the different opioid receptors involved in opioid-mediated insulin secretion and glucose homeostasis may lead to the identification of new target strategies in diabetes therapy. A major step towards this aim is to understand the individual role of the classical opioid receptors in insulin secretion.

Here, I report the effect of different opioid receptor-selective ligands, agonists and antagonists, to glucose-induced insulin release in a pancreatic β-cell line. In this study, I am using the well-characterised pancreatic β-cell line, RIN-5F, which releases insulin in glucose-containing media (534).

5.2. Methods

5.2.1 Materials

The pancreatic β-cell line (RIN-5F) was purchased from the American Type Culture Collection (ATCC® CRL-2058™; P.O. Box 1549 Manassas, VA 20108 USA). The insulin immunoassay kit was purchased by Merck Millipore (EZRMI-13K; Massachusetts 01821, USA). All tissue culture media and supplements were obtained from Life Technologies (GIBCO®, Paisley, U.K.). [D-Pen2|5]-enkephalin (DPDPE), nor-binaltorphimine (nor-BNI) and the D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) were purchased by Abcam Biochemicals. The [D-Ala2 N-Me-Phe4 Gly5-ol]-enkephalin (DAMGO) was purchased by AdipoGen. The (-)-U-50488 hydrochloride (U50488) was purchased by Tocris Bioscience. Naltrindole hydrochloride and the trypan blue solution (0.4%,100 ml/bottle) were purchased by Sigma-Aldrich. All other reagents used were purchased with the highest quality available.

5.2.2. Cell culture and treatment

Cells were cultured and maintained in a similar way as previously described (349). Briefly, RIN-5F rat pancreatic β-cells were grown in 25cm2 flasks in RPMI-1640 media containing L-glutamine, substituted with 10% heat-inactivated foetal calf serum, 100 IU ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin. Stock cultures were grown at 37° C with 5% CO_2 95% humidity and were sub-cultured twice a week as required using phosphate buffered saline and trypsin/0.5mM EDTA. Cells were used for experiments as they approached confluence. An appropriate volume of cells was seeded in the wells of a 96-well plate, in the presence or absence of drugs in a total volume of 200µl and was incubated for 24 hours. Prior to the main experiment, the cell number-dependent basal insulin secretion levels of RIN-5F cell line($1x10^4$, $2x10^4$ and $4x10^4$ cells/well) were determined.

5.2.3. Drug treatment

Cells were treated with a selective opioid-receptor agonist at 1μM final concentration (DAMGO, a MOP agonist; DPDPE, a DOP agonist; U50488, a KOP agonist) to determine their effect on insulin secretion (n = 4 - 5 per ligand). A combination treatment of an opioid agonist plus the respective opioid-receptor antagonist at 10μM final concentration (CTOP: a MOP antagonist, naltrindole: a DOP antagonist, nBNI: a KOP antagonist) was used in some wells to determine if the potential effect of the agonist was receptor-mediated (n = 4 - 5 per group). Antagonists where used at a higher concentration than the agonist in order to ensure sufficient displacement of the agonist from their receptor binding site. In addition, cells in some wells were also treated with an antagonist alone (n = 4 - 5 per ligand), to determine the effect of these ligands on insulin secretion. Cells that were not treated with any compound were used as a negative control.

5.2.4. Immunoassay for insulin detection

An enzyme-based immunoassay (sandwich ELISA) was used to detect the amount of insulin secreted by RIN-5F cell line, in the presence or absence of test compounds, according to the manufacturer's instructions. In brief, after incubation of cells with test compounds for 24 hours, a supernatant sample of 10μl was taken from all wells and transferred to a microtiter plate pre-coated with a monoclonal mouse anti-rat insulin antibody. The captured insulin was then detected by a second, biotinylated polyclonal anti-insulin antibody. Unbound materials were thoroughly washed away with manufacturer's washing buffer prior to the binding of streptavidin-horseradish peroxidase to the immobilised biotinylated antibodies. Another step of thorough washing of free enzyme conjugates followed before the final quantification of immobilised antibody-enzyme conjugates. The quantification was performed by monitoring the horseradish peroxidase activity in the presence of its substrate (3,3',5,5'-tetramethylbenzidine).

The enzyme activity was measured by spectrophotometry at 450 nm, corrected from the absorbance of 590nm, after the acidification of formed products with 0.3M HCl. Since the increase in absorbance is directly proportional to the amount of captured insulin in the samples, the latter was derived by interpolation from a reference curve generated within each performed assay using reference standards of known concentrations of rat insulin. The dose-response curve produced by the internal standards was fitted to a sigmoidal 4-parameter logistic equation according to the manufacturer's instructions by plotting the corrected absorbance at 450nm minus 590nm, against the concentration of rat insulin standards.

5.2.5. Viability assay

A trypan blue exclusion assay was used to determine the viability of the pancreatic cells in the presence of test compounds, according to the Strober method (535). Trypan blue is impermeable to intact healthy cells but can enter cells with a compromised cell membrane creating an intracellular blue staining that can be detected by light microscopy. Trypan blue staining can be therefore used as a marker of cell death. Cells treated for 24 hours with test compounds were trypsinised, stained with 0.04% trypan blue. Before the number of stained cells versus the total number of cells was counted using a haemocytometer under a light microscope. Viability was defined as viable cells per well.

5.2.6. Data analysis

Concentration-response curves were analysed by non-linear regression or by a 4-parameter sigmoidal fit, as appropriate. GraphPad Prism V6.0 software (San Diego, CA, USA) was used for statistical analysis. Students t-test and analysis of variance (ANOVA) with multiple comparison testing were used as required and as described in the table and figure legends (minimum significance set at $p<0.05$). Data are presented as mean ± SD from (n) number of

experiments as indicated in the respective figure or table legends.

5. 3. Results

5.3.1. Optimisation of insulin detection

RIN-5F cell line physiologically secretes insulin when incubated in an appropriate glucose-contained growth medium. Since the amount of secreted insulin/well is dependent on cell numbers, I first optimised cell numbers per well for a 96 well format to obtain absorbance values that fall within the range of values of the standard curve. Three different concentrations were used ($1x10^4$, $2x10^4$ and $4x10^4$ cells/well) with five parallel wells per group.

Known amounts of insulin (0, 0.5, 1, 2, 5, 10 ng/ml) were used in triplicates as internal standards to produce a standard curve (Fig. 31), which was initially fitted using linear regression analysis ($R^2 = 0.9898$), producing a significant slope (0.1191 ± 0.004; $p<0.0001$) and confirming the linearity of the internal standards (Fig. 31 A). Subsequently, the same data was fitted more efficiently to a sigmoidal 4-parameter logistic equation (Figure 31 B; R^2 0.9990, EC_{50} 5.62, HS 0.23), which did not produce any significant normality of residuals (D'Agostino & Pearson's test; $p = 0.583$). These data confirmed the manufacturer's suggestion for using the sigmoidal fitted curve for interpolation of data derived from samples. Absorbance values from the three cell concentrations tested were plotted against the derived insulin values from the sigmoidal standard curve (Fig. 31 C). Although all three groups produced large amounts of secreted insulin (> 20 ng/ml) using 10^4 cells per well produced the least variability (variation coefficient 2.80 %) and a mean absorbance value of 2.65, which translated to 23.8 ng/ml of insulin per well. Since the minimum acceptable density for optimum growth of the RIN-5F cell line in a 96-well is 10^4, this cell density was chosen to be used for all subsequent experiments. The rationale was that the collected samples of these wells would be diluted 4 times before processing by ELISA and therefore the secreted basal insulin value would be expected to fall within the EC_{50} of the curve (e.g. 23.8 $ng.ml^{-1} \div 4 = 5.95 \approx EC_{50}$ 5.62).

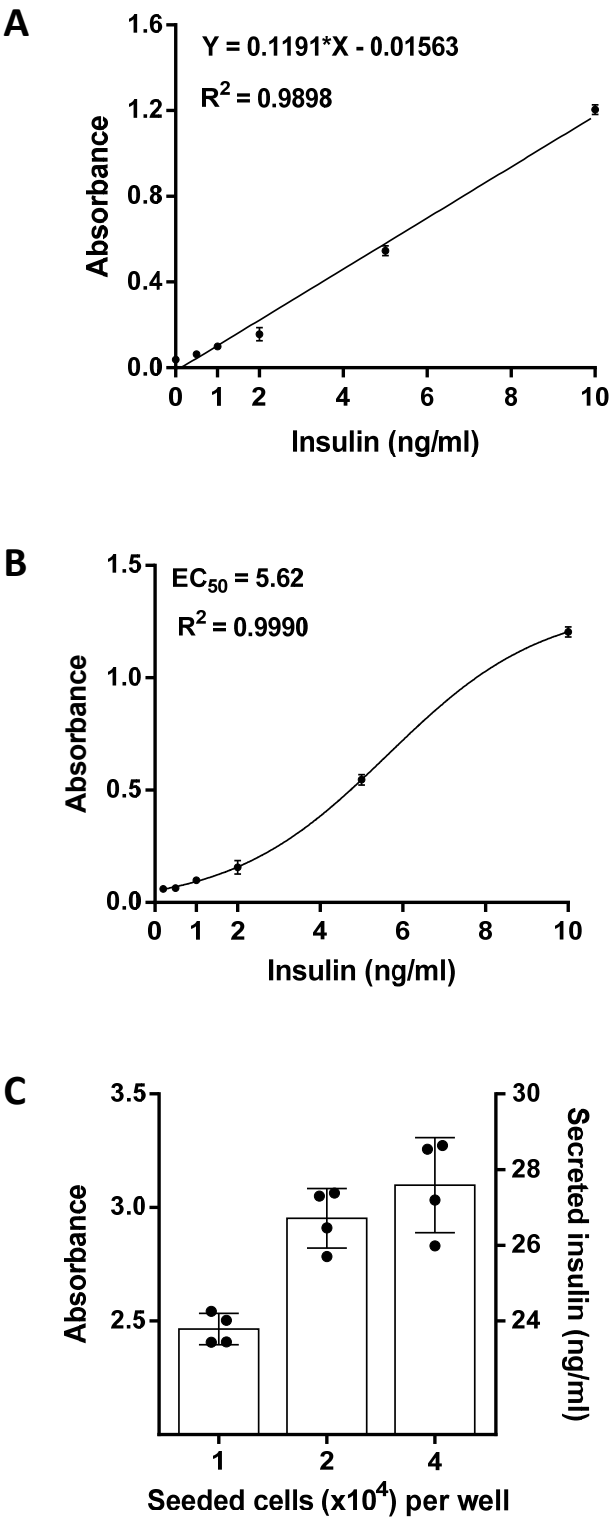


Figure 31. Optimisation of the insulin detection method using an enzyme-based immunoassay (ELISA). A standard curve was generated by using known amounts of insulin (0, 0.5, 1, 2, 5, 10 ng/ml) in triplicates which were fitted using linear regression analysis (A) producing a significant slope (0.1191 ± 0.004; $p < 0.0001$) and confirming the linearity of the internal standards. The same data were fitted more efficiently into a sigmoidal 4-parameter logistic equation (B) which did not produce any significant normality of residuals (D'Agostino & Pearson's test; $p = 0.583$). Three different cell densities ($1x10^4$, $2x10^4$ and $4x10^4$) were seeded in individual wells and the corresponding standard curve was used to calculate the total amount of secreted insulin per well (C). Absorbance was measured at 450 nm corrected for the absorbance at 590 nm. All data are shown as mean ± SD from n = 4 - 5.

5.3.2. Total secreted insulin after opioid treatment

After determining the optimal cell density numbers per well, I measured insulin secretion of cells seeded and treated with test compounds. An internal standard curve was generated within this assay and analysed using both analysis methods (linear regression and the 4-parameter sigmoidal logistic equation; Fig. 32 A, B). In agreement with the previous data, the sigmoidal standard curve provided a better fit compared to a linear fit and was therefore used to interpolate the absorbance values produced by the samples.

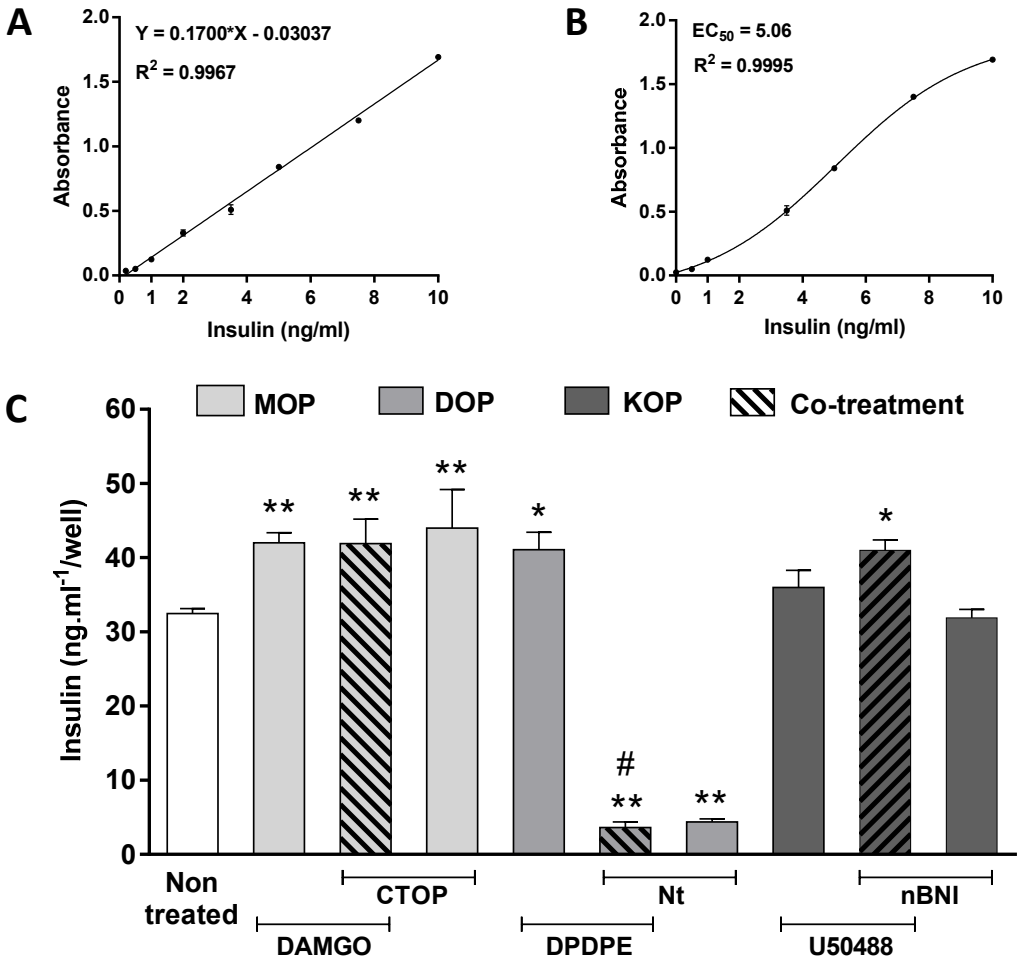


Figure 32. Detection of total insulin secreted in the well, using an enzyme-based immunoassay (ELISA). A standard curve was generated by using known amounts of insulin (0, 0.5, 1, 2, 3.8, 5, 7.8, 10 ng/ml) in triplicates which were fitted using linear regression analysis (A) and a sigmoidal 4-parameter logistic equation (B); the latter used to calculate the total insulin concentration in wells treated or non-treated with opioid-receptor selective ligands (C). Absorbance was measured at 450 nm corrected for the absorbance at 590 nm. One-Way analysis of variance (ANOVA) was used with a Sidak multiple comparison test (* $p < 0.005$ and ** $p < 0.0005$ compared to non-treated control; # $p < 0.0001$ compared to DPDPE). All data are shown as mean $\pm$ SD from n = 4 - 5.

After taking into account the dilution factor used to dilute the samples (x 4), the concentration of secreted insulin by the seeded cells was calculated and expressed as a bar-chart (Fig. 32 C). Compared to the untreated control, treatment of cells with the selective MOP receptor ligands DAMGO, CTOP and combination of both, significantly increased insulin secretion. Treatment with the DOP receptor-specific agonist DPDPE also significantly increased insulin secretion. A more peculiar relationship was observed for the KOP receptor, where the selective agonist U50488 and the antagonist nBNI did not elicit any effects, while conversely their combination significantly increased insulin secretion (Fig. 32 C). Finally, naltrindole-treated cells (with or without DPDPE) showed a highly significant decrease of insulin secretion, reflecting the high-toxicity of naltrindole that I previously observed in non-pancreatic cell line (non-published observation).

5.3.3. Secreted insulin/cell numbers at endpoint

Since the test compounds can potentially affect cellular viability and proliferative capacity, which is likely to affect the extent of secreted insulin, it is important to account for these potential effects when evaluating the stimulatory or inhibitory effects of drug-induced insulin secretion. I, therefore, measured cellular viability using trypan-blue (TB) exclusion, immediately after the collection of the supernatant. Since the assay provides information on the number of dead and alive cells in each treated well, it allows the expression of the total results as a ratio of secreted insulin/live cells at the endpoint. Taking the number of live cells in the non-treated wells as a baseline, I calculated the live cells per treated well. Different opioid treatments differentially affected cell viability and proliferation over the same time-period (Table 22). Consistent with our previous results (unpublished), TB staining revealed that naltrindole was highly toxic to pancreatic RIN-5F cell line, with no viable cells after 24-hour incubation with this DOP-selective antagonist.

Table 22. Cell viability data from cells treated with different opioid ligands compared to non-treated control using a trypan-blue exclusion assay. Results are expressed a mean number of cells viable per well after 24 h treatment (± SD from n = 4 - 5). One-way analysis of variance (ANOVA) was used with a Sidak multiple comparison tests (*p < 0.005 and **p < 0.0005 compared to non-treated control).

Treatment	Number of viable cells per well (x 10^2; mean ± SD)
No ligand (control)	520 ± 80
DAMGO	346 ± 166
DAMGO + CTOP	520 ± 153
CTOP	410 ± 167
DPDPE	370 ± 9
DPDPE + naltrindole	120 ± 15 *
Naltrindole	0 **
U50488	430 ± 92
U50488 + nBNI	530 ± 92
nBNI	320 ± 16

After determining the number of viable cells per well after 24 hours of treatment, and standardised insulin concentrations on cell numbers (Fig. 33). The insulin values of Fig. 33 are shown in Table 23, as mean insulin secretion and as a percentage increase or decrease compared to the baseline control. Untreated control cells produced insulin levels of 2.24 (± 0.85) ng/ml/10^3 cells, while in comparison the MOP receptor agonist DAMGO nearly doubled the measured insulin levels (12.12 ± 0.81 ng/ml/10^3 cells; a 94.2 % increase compared to control). In parallel, the DOP receptor agonist DPDPE also significantly stimulated insulin secretion (11.00 ± 2.55 ng/ml/10^3 cells; a 76.3 % increase compared to control). Interestingly, compared to DPDPE the MOP receptor antagonist CTOP on the other hand, also prolonged insulin secretion (10.73 ± 3.11 ng/ml/10^3 cells; a 72 % increase). Co-treatment of CTOP and DAMGO

was able to reduce the effect of their individual administration, although showing a trend towards increased insulin secretion, which was not significant from control. Contrary to the MOP and DOP ligands, none of the KOP agonist and antagonist tested significantly affected insulin levels, despite a small trend towards higher insulin levels.

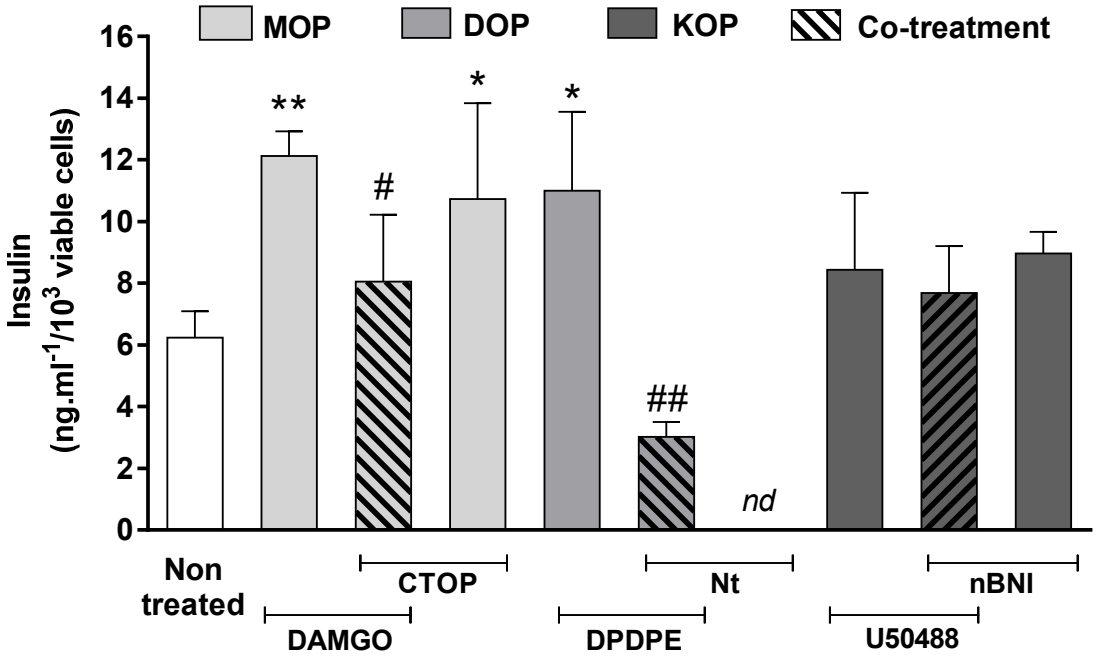


Figure 33. Detection of insulin secreted per 10^3 viable cells by using an enzyme-based immunoassay (ELISA). The absorbance recorded as the differences of absorbance from 450 nm minus 590 nm. The chart was produced by combining the total insulin data per well from Figure 32 C and the cell viability data from Table 22. One-Way analysis of variance (ANOVA) was used with a Holme-Sidak multiple comparison test (* $p < 0.05$ and ** $p < 0.005$ compared to non-treated control; # $p < 0.05$ compared to DAMGO and ## $p < 0.0005$ compared to DPDPE). All data are shown as mean $\pm$ SD from n = 4 - 5. Key: nd, not detected.

Table 23. Secreted insulin concentration per viable cells after treatment with opioid-receptor selective ligands, compared to basal (non-treated control). Concentration data are extracted from Figure 33 and are shown as a percentage of significant change from the control. One-Way analysis of variance (ANOVA) was used with a Holme-Sidak multiple comparison test (* $p < 0.05$ and ** $p < 0.005$ compared to non-treated control; # $p < 0.05$ compared to DAMGO and ## $p < 0.0005$ compared to DPDPE). All data are shown as mean ± SD from n = 4 - 5. Key: nd, not detected.

Treatment	Secreted insulin (mean ng/ml/10^3 viable cells ± SD)	Significant % change from control
No ligand (control)	6.24 ± 0.85	-
DAMGO	12.12 ± 0.81	+ 94.2 (**)
DAMGO + CTOP	8.06 ± 2.17	- (#)
CTOP	10.73 ± 3.11	+ 72.0 (*)
DPDPE	11.00 ± 2.55	+ 76.3 (*)
DPDPE + naltrindole	3.02 ± 0.48	- 51.6 (##)
Naltrindole	nd	nd
U50488	8.43 ± 2.49	-
U50488 + nBNI	7.68 ± 1.53	-
nBNI	8.96 ± 0.70	-

Finally, since naltrindole completely killed the treated cells over a period of 24 hours, the ratio of insulin release/cell number could not be determined. However, the total insulin reduction in the naltrindole-treatment shows that naltrindole's toxicity is so rapid that it inhibited the basal glucose-induced insulin excretion. In addition, the increase of insulin seen with DPDPE was significantly abolished by naltrindole co-treatment (3.02 ± 0.48 ng/ml/10^3 cells; a 51.6 % decrease) due to naltrindole's toxicity, but it was not to zero. DPDPE's concentration in the co-treatment group was 10 % of that of naltrindole and was still able to contain some of naltrindole's toxicity, insinuating a competitive behaviour for both actions (DPDPE's insulin stimulation and naltrindole's toxicity) which most likely is mediated through the DOP receptor.

5.4. Discussion

Opioids represent an important drug class in diabetic pharmacotherapy due to their role in diabetic neuropathy and their complex and under-explored endocrine effects. Although opioids are not considered very effective as a stand-alone treatment for diabetic neuropathic pain, their strong analgesic effect offers a potential co-administration solution to drug-resistant and persistent neuropathic pain (536). In addition, the remarkably wide range of tissues that express the classical opioid receptors constitutes the basis of their non-analgesic side effects, some of which are currently explored for conditions other than pain (537,538,539). The expression of opioid receptors in pancreas and liver (537) initiated early studies to investigate the role of endogenous opioids in processes associated with the endocrine and autocrine system (540), such as glucose homeostasis (512) and insulin release (533), as well as in related conditions such as diabetes (541), obesity (542), exercise-associated autonomic failure (543), hypoglycemia-associated failure (388) and polycystic ovary syndrome (544) among others.

Since insulin plays a central role in glucose homeostasis, in pancreas autocrine function and in diabetes, the effect of opioid ligands (endogenous and synthetic) on glucose-induced and glucose-independent insulin secretion has attracted significant attention. However, the convoluted relationship between different feedback systems in pancreatic endo-/autocrine function, as well as the different pharmacology of the three classical opioid receptor types, has created some inconsistency in the literature and a certain level of complexity in the interpretation of published data. The main question posed was: do opioids increase or decrease glucose-induced insulin secretion? Answering this question is not a simple task, given the different cell types in the pancreas, the effect of glucagon and other peptides on pancreatic function, the cross-talk between liver and pancreas on glucose homeostasis and the central nervous system effect on the pancreas (e.g. insulin secretion and opioidergic neuron activation).

The majority of these studies on opioids and insulin secretion have directed on the effect of endogenous opioid ligands (e.g. enkephalins, endorphins) or clinically used opioids (e.g. morphine, fentanyl) on insulin secretion in isolated pancreatic islets. To the best of my knowledge, there are no current data in the literature regarding opioid receptor-specific agonists and antagonists and their effect on insulin secretion in a single study. In this thesis, I provide the first data for MOP, DOP and KOP selective agonists and antagonists in terms of their effect on insulin secretion in pancreatic β-cell line (RIN-5F) (Fig. 34). RIN-5F cell line was originally generated from tumours of islets of Langerhans of male rats and was purchased from the American Type Culture Collection (as described in the section 5.2.1 of this thesis) (545). MOP receptors are expressed in the islets of Langerhans of mice and their expression has been reported to regulate glucose homeostasis (546). For example, MOP receptor (MOP-1) knock-out mice showed increased glucose tolerance, which resulted from enhanced insulin secretion from β-cells (546). The expression of the DOP and KOP receptors were also verified for the islets of Langerhans in rats (547). Therefore, there is the possibility that the RIN-5F cell line could also express the MOP, DOP and KOP receptors similar to the original tissue. However, due to a lack of data regarding the expression of specific opioid receptors in RIN-5F cells, I have no evidence that all opioid receptors are actually expressed in this cell line. This is an obviouslimitation of my current study and should be investigated in future studies.

Our outcomes provide preliminary evidence that MOP and DOP selective ligands DAMGO and DPDPE respectively can stimulate overall glucose-induced insulin secretion from a β-cell line, measured after 24 hours of treatment; a time-period which would include both phases of insulin's biphasic secretion. These data fit with the majority of the previous studies on opioid-mediated insulin release, which show a stimulatory effect of opioids as described in the introduction section above.

Similarly to Qian et al (402), who used an MTT viability assay to assess whether opioid effect cellular toxicity of the opioid concentrations used, I used the TB viability assay to account for the potentially different effects of the different opioids used.

In addition, I have observed that the MOP-selective antagonist CTOP also significantly increased insulin secretion in our cells, which clearly shows that a MOP receptor antagonist can also increase insulin secretion similarly to MOP receptor agonist like DAMGO. The increased insulin secretion is presumably mediated either through a non-opioid receptor mechanism or through a G-protein-activation independent mechanism, but such measurements were out of scope of the current study. However, the reduced effect on insulin release by the co-treatment of DAMGO and CTOP compared to individual treatment presents a case of antisynergism, which insinuates an opioid-receptor mediated mechanism, although further investigation is needed to draw solid conclusions.

In this thesis, I also show the insignificant contribution of the KOP receptor-specific opioids to insulin secretion, as shown by the non-significant change of insulin secretion by the KOP receptor agonist U50488 and the KOP receptor antagonist norBNI. Finally, I show in this thesis that the DOP receptor selective antagonist naltrindole possesses a significant toxicity to β-cells, which seems to be partially attenuated by the co-administration of the DOP receptor selective agonist DPDPE. Whether this effect of DPDPE is interpreted as a protection against naltrindole's toxicity, or simply a result of a competition for DOP receptor binding, the conclusion drawn is similar.

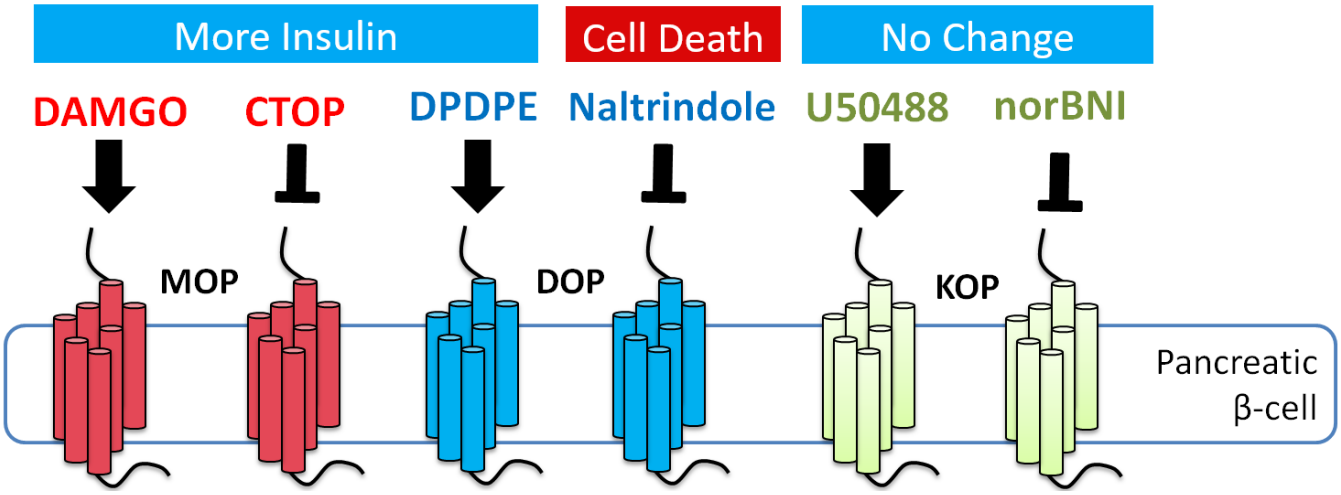


Figure 34. Schematic illustration of the effects of different opioid-receptor selective opioid ligands on insulin secretion and cell viability in pancreatic RIN-5F β-cell line. Ligands (agonists at 1 µM; solid arrow, antagonists at 10 µM; blunt arrow) on insulin secretion of a pancreatic β-cell line, as generated from this study. Both the agonist and the antagonist targeting the MOP receptor produced an increased insulin secretion compared to control, similar to the DOP receptor selective agonist. Naltrindole was highly toxic, whereas none of the KOP receptor selective ligands produced a significant change in insulin levels compared to control.

Since the present data suggest that insulin release can be increased by both agonists and antagonists of the MOP and DOP receptors (e.g. DAMGO, DPDPE, CTOP), it intrigued us to hypothesise an opioid-mediated insulin secretion which is dependent on opioid receptor-binding but being independent of G-protein activation. This hypothesis fits with a study by Green et al (395) that ascertained a cAMP-independent mechanism of opioid-induced stimulation of insulin secretion, as well as with data from Olianas et al (391) who observed a cAMP-independent mechanism of DPDPE-induced glucose uptake. DPDPE and DAMGO are both analogues of enkephalin (Tyr-Gly-Gly-Phe-Met/Leu) that possess different selectivity on opioid receptors (DOP and MOP receptors respectively), while CTOP is also a tyrosine and penicillamine containing peptide. It is, therefore, possible that these structurally similar ligands bind to MOP or DOP receptors and cause a cAMP-independent intracellular reaction that causes insulin-containing vesicles in β-cells to release their insulin.

The present study suggests that the KOP receptor targeted ligands U50488 and norBNI did not produce a significant insulin release compared to control. These two ligands are structurally very different from the rest of the opioids tested since they are not peptides. In addition, although it is questionable whether KOP receptors are expressed in pancreatic islets, higher concentrations of these ligands need to be tested in the future to check whether there is an effect on insulin secretion. However, in a recent study by Shang et al (372), treatment of STZ-mice with U50488 reduced hyperglycaemia but did not affect plasma insulin levels, which shows that KOP receptors may play a role in glucose-homeostasis rather than insulin secretion per se. Previous studies show that U50,488 (1-100 100 μM) can block neuronal voltage-gated sodium channels and produce visceral antinociception in rats, which indicates a KOP receptor independent effect (548,549). Other reports indicate that blockage of sodium channels in human β-cells can reduce glucose-stimulated insulin secretion and microRNA-375 can regulate the voltage-gated sodium channels in β-cells (550,551). A sodium channel activator (BDF-9148) has shown to inhibit insulin secretion, whereas a sodium channel blocker (carbamazepine) protected β-cells from cytokine-induced cell-death (552,553). Voltage gated potassium channel Kv2.1 activation induces efflux of potassium ions from pancreatic β-cells and reduces glucose-stimulated insulin secretion in these cells, as Kv2.1 knockout mice (-/-) showed increased glucose-stimulated insulin secretion compared to the pancreatic β-cells of wild-type mice (554). Sulphonylureas block ATP-sensitive potassium channels and activate insulin secretion and is widely used to manage diabetes mellitus type 2 (T2DM) (555). Together a multifaceted network regulates insulin section and includes opioid receptors as well as several ion channels.

5.5. Conclusion

My study furnishes evidence of the opioid-induced increase of insulin secretion, by receptor-selective agonists and antagonists, which implies that the role of opioid receptors in insulin secretion in β-cell line is more complicated than G-protein dependent. Moreover, an investigation into the molecular pathways that are involved downstream the opioid receptor after ligand binding is needed to clarify the role of opioid receptors on insulin secretion. Understanding these pathways may provide an insight into novel pancreatic mechanisms of insulin secretion and the identification of novel drug targets for diabetes. My study shows that MOP receptor agonist increases insulin secretion in a pancreatic β-cell line, which is in agreement with clinical studies that show that these compounds reduce blood glucose levels (381,382,383,384,385,386). Therefore, the clinical significance of the effects of opioids on glucose or insulin sensitivity can be extrapolated from my *in vitro* study.

CHAPTER SIX

In vitro characterisation of novel opioids

Preface to chapter six

In the previous chapters (*chapters one to five*), I observed that morphine induces different adverse effects by interacting with both opioid and non-opioid receptors. This illustrates the need for better pharmacological alternatives to reduce the impact of the adverse effects of morphine. Previous studies showed that opioid ligands with mixed activity on different opioid receptors display reduced adverse effects compared to morphine. Different novel opioids were synthesised at the University of Tasmania and were investigated for their selectivity towards different opioid receptors. This chapter describes the pharmacological characteristics of a range of novel opioids (*chapter six*) to identify the best ligand for further preclinical investigations.

6. *In vitro* characterisation of novel opioids

Abstract

Opioids with mixed activity on different opioid receptor are known to be associated with less adverse effects than morphine or other clinical opioids. In particular, Dmt-Tic pharmacophore-based opioids such as UFP-505 show MOP receptor agonist / DOP receptor partial agonist activity. In this study, I evaluated six ligands based on Dmt-Tic peptides. These compounds were tested for their receptor selectivity using different opioid receptor expressing Chinese Hamster Ovary (CHO) cells. For the most promising of these six ligands, termed UTA1003, dose-responses were established to determine its median effective concentration (EC_{50}). Overall, UTA1003 showed efficacy as a MOP and KOP receptor agonist with a DOP receptor partial agonist, with better solubility and a similar pharmacological profile compared to the parental compound UFP-505. Therefore, UTA1003 represents an improvement over its parental compound and might be a starting point for a rational medicinal chemistry program to develop antinociceptive drugs without the severe adverse effects of morphine.

Keywords: UFP-505; UTA1003, Dmt-Tic; opioid; MOP receptor agonist; DOP receptor.

6.1. Introduction

Selectivity of novel opioids on multiple opioid receptors has been the focus of opioid research in recent years, due to the evidence of reduced antinociceptive tolerance and/or behavioural side-effects in different preclinical studies (160,178,337). A combination of an opioid receptor agonist (e.g. morphine) and opioid receptor antagonist (e.g. naltrindole, naltrexone) have shown better antinociception and less tolerance than an opioid agonist alone (122,123,124,125,278,279). However, compounds with selectivity on multiple opioid receptors may reduce the possibility of drug-drug interactions and improved pharmacokinetic or pharmacodynamic (PK/PD) profile than a drug combination (280,281,282). Many of these ligands act simultaneously on different opioid receptors (282,283) or a combination of an opioid receptor and a non-opioid receptor (284,285,286). Therefore, ligands with selectivity on multiple opioid receptors show better antinociception, less antinociceptive tolerance and other behavioural side-effects than the current clinical opioids (151,178,280,281,337). As a result, a potential strategy in recent years to avoid both antinociceptive tolerance and behavioural side-effects could be the proposed use of a single mixed-selective compound and has been a topic of interest in the area of opioid pharmacology.

Opioids with selectivity on multiple receptors are different in their structures, such as peptides (306,346,556), peptidomimetics (312,348,353,557) and alkaloids (558). Importantly, ligands with mixed activity on multiple receptors, such as UFP-505 and DIPPψNH$_2$, showed reduced antinociceptive tolerance and behavioural side-effects than morphine (346,354). However, the potential clinical utility of these compounds is limited. DIPPψNH$_2$ showed poor blood-brain barrier penetration and produced seizures after administration of a high dose (10-400 µg i.c.v.) (282,346). Similarly, UFP-505 was reported to induce antinociceptive tolerance and toxicity after intracerebroventricular (i.c.v.) administration, but it did not induce these adverse effects

after intrathecal administration (306,354). Importantly, UFP-505 did not appear to induce its antinociceptive effects after systemic (subcutaneous or intravenous) administration (354). (349,350,559)

As a part of ongoing chemical and preclinical investigations on opioids with selectivity on multiple receptors, I am describing different novel UTA-opioids based on the prototype UFP-505 (H-Dmt-Tic-Gly-NH-Bzl) (Fig. 35). The current study has focused on different human opioid receptors in order to interpret the detailed pharmacological effects using recombinant human MOP, DOP, KOP or NOP receptors expressing Chinese Hamster Ovary (CHO) cells. Measurement of G-protein activation (or blockage) following a ligand binding to a G-protein coupled receptor (GTPγ^{35}S binding assay) or measurement of its effects in downstream pathways, such as ligand-binding induced changes in cellular cAMP levels (cAMP assay), are typically used to characterise novel opioids (160,178,349,560). This study used the cAMP assay as a tool to characterise the specificity of UTA-ligands using MOP, DOP, KOP or NOP receptors expressing CHO cells. This approach was designed to select the best ligand with a full MOP receptor agonist / DOP receptor antagonist (or a partial agonist) profile out of these six UTA-opioids for subsequent preclinical studies.

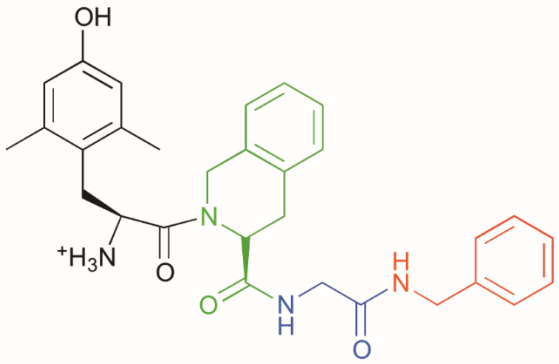


Figure 35. Chemical structure of UFP-505 (H-Dmt-Tic-Gly-NH-Bzl). Key: Dmt: 2',6'-dimethyl-l-tyrosine, Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, Gly: Glycine, Bzl: Benzyl.

6.2. Materials and methods

6.2.1. Materials

Cellular cAMP levels were measured in CHO cells with recombinant expression of different types of human opioid receptors. Wild-type CHO cells without recombinant opioid-receptor expression were also used to investigate whether the measured effects are associated to specific opioid receptors. The Promega cAMP-Glo^{TM} Max assay kit (V1681, Promega Corporation, Madison, USA) was used in this study. The commercial kit contained cAMP 100 mM cAMP, 1 M $MgCl_2$, cAMP-Glo^{TM} ONE-buffer, Protein Kinase A, Kinase-Glo® lyophilised substrate and Kinase-Glo® buffer. In addition, IBMX (3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione) and Ro20-1724 (4-(3-Butoxy-4-methoxyphenyl)methyl-2-imidazolidone), Forskolin ([3*R*-(3α,4aβ,5β,6β,6aα,10α,10aβ,10bα)]-5-(acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1*H*-naphtho[2,1-*b*]pyran-1-one) were purchased from Tocris Bioscience, Bristol, UK. IBMX and Ro20-1724 are phosphodiesterase inhibitors to prevent hydrolysis of cAMP (561). Forskolin as positive control activates adenylyl cyclase enzymes and increases intracellular levels of cAMP (562). IBMX and Ro20-1724 were diluted to 100 mM solution using 100% DMSO. The solution was further diluted to 500 µM IBMX and 100µM Ro20-1724 using PBS. Afterwards, complete induction buffer was prepared using 500 µM IBMX and 100µM Ro20-1724 and 25 mM $MgCl_2$. The solution was used to see basal cAMP expression of cells in the wells without treatment. Forskolin was diluted to 200 µM using DMSO, which was further diluted to 1- 50 µM with freshly prepared complete induction buffer immediately before the experiments. Forskolin was used as a positive control and all other UTA-ligands and standard compounds were diluted in forskolin solution. The cAMP detection solution was freshly prepared using 1:100 ratio of PKA and cAMP-Glo buffer (as stated in the supplier's guide). Kinase-Glo® buffer and substrate was mixed and it was called as Kinase-Glo reagent. The kinase-Glo reagent was stored at -20°C as 1 ml aliquots. Required

amount of kinase-Glo reagent aliquots were thawed at room temperature and used immediately. UTA- ligands were dissolved at 10 mM with 100% DMSO and the solution was further diluted to the required concentrations using diluted forskolin solution.

6.2.2. Chemical synthesis

The analogues of UFP-505 have been synthesised as a drug optimisation study and described in a previous report (563).

6.2.3. cAMP assay

CHO cells were grown in T-25 flasks containing F-12(Ham) media (1x) for the wild-type, MOP, DOP, KOP and NOP cells containing 10% foetal bovine serum and 100 IU/ml penicillin. All media contained L-glutamine. The cell culture media were additionally supplemented with 200 mg/ml G418 (selection agent used with MOP, DOP, and KOP cells) and with additional 200 mg/ml hygromycin B for the NOP cells once in every four passages. Cell cultures were kept at 37^{o}C 5% v/v CO_2 and 95% v/v humidified air. Trypsin/EDTA was used as the minimum required to split the cells during sub-cultures. The cells were used for experiments, as they were approached confluence.

The assay was conducted using the supplier's protocol. In short, the CHO-cells were seeded as 8000-10000 cells/well in 96-well plates and incubated for 24 hours. On the following day, all reagents were thawed and prepared as per the supplier's guidelines. Firstly, after removing media using an aspirator, cells were incubated in 40 µl of complete induction buffer (blank), no drug (forskolin 1-50 µM), UTA-opioids and reference drugs for 20 min at 37^{o}C (incubator). Secondly, 10 µl freshly prepared cAMP detection solution was added to all wells and mixed by a plate shaker for 2 min. The plate was further incubated for 20 min at room temperature

(22-23°C). Afterwards, 50 µl kinase-Glo reagent was added to each wells and mixed by a plate shaker for a minute and the plate was incubated for 10 min at room temperature. Finally, 60 to 80 µl solutions from each well were transferred carefully to a white round bottom plate and immediately put into a plate-reader to measure luminescence. The measurement was repeated twice (after 1 min of first measurement) to verify the luminescence signals. Each drug was tested using six different wells and the average value was used for statistical analysis.

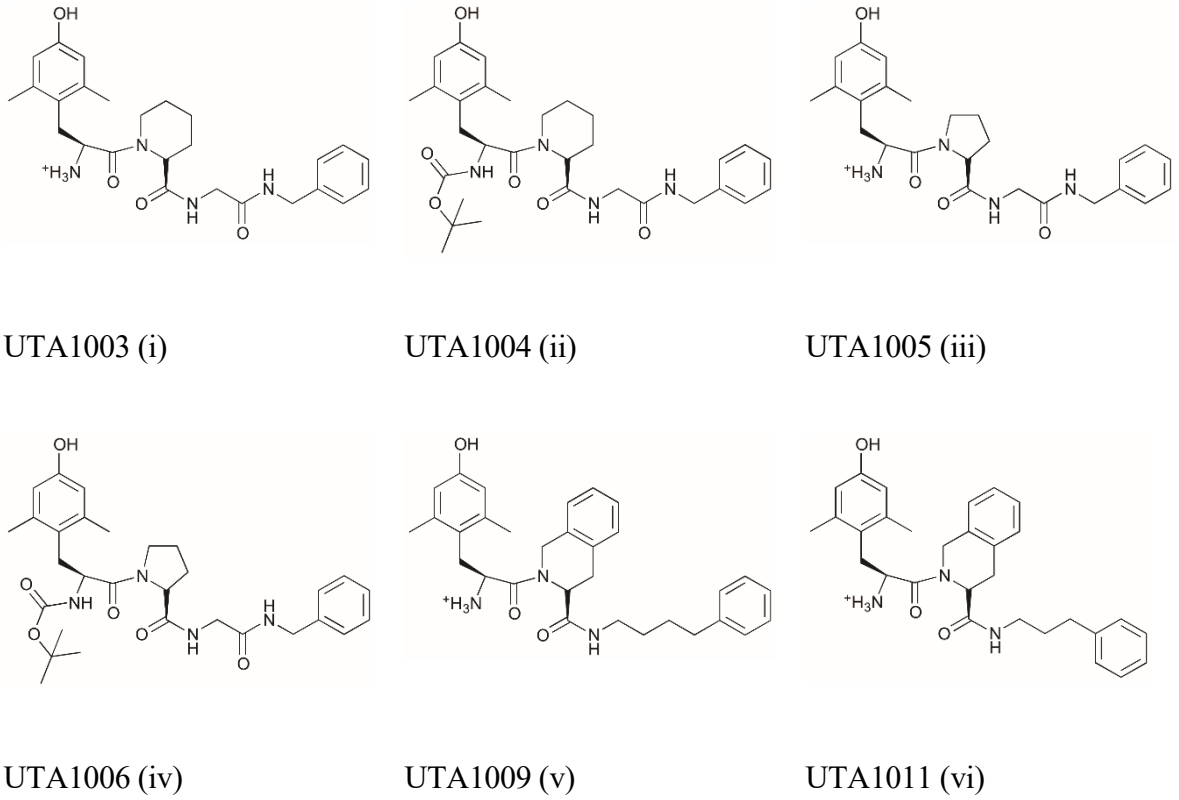
6.2.4. Calculations and statistical analysis

For a direct comparison between the UTA ligands, standard compounds and for the concentration response curves, the pharmacological effect was calculated as % of inhibition of forskolin-induced cAMP production and the values were used for statistical analysis. Antagonist effects of UTA1003 were calculated as follows, K_d = [opioid]/(CR-1), where CR is the ratio of the EC_{50} of DPDPE in the presence and absence of UTA1003, [opioid] is the concentration of UTA1003 used with DPDPE in the antagonism test (160,560). All data are expressed as mean ± SEM (n = 6) and analysed by one-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism V6 software (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons (Dunnett's test) was employed when F achieved $p < 0.05$ and there was no significant variance in homogeneity. A 'p' value less than 0.05 was considered statistically significant.

6.3. Results

6.3.1. Structures of novel UTA-opioids

In this study, six analogues of UFP-505 were generated with the objective to increase their solubility and also to sequentially reduce their peptidic structure (Fig. 36). These chemical changes were hypothesised to improve the ADME characteristics and metabolic stability of the compounds while retaining their antinociceptive effects. The detailed synthesis of the six novel UTA-ligands (Fig. 36) has been described in detail in a previous study (563).



UTA1003 (i) UTA1004 (ii) UTA1005 (iii)

UTA1006 (iv) UTA1009 (v) UTA1011 (vi)

Figure 36. Chemical structures of six UTA-opioids

6.3.2. Specificity of novel UTA-opioids on different opioid receptors

The specificity of the six UTA ligands for different opioid receptors was measured using several recombinant human opioid receptors expressing Chinese Hamster Ovary (CHO) cell-lines. Firstly, the ligands were evaluated for their effects on untransfected Chinese Hamster Ovary (CHO-WT) cells to confirm that the UTA-ligand mediated effects are opioid receptor specific (Fig. 37). Using this assay, UTA1006, UTA1009 and UTA1011 had a slightly but significant ($p < 0.01$) inhibitory effect on forskolin-stimulated cAMP production (Fig. 37). This effect was not observed for UTA1003-1005, UFP-505 and DAMGO (a MOP receptor agonist).

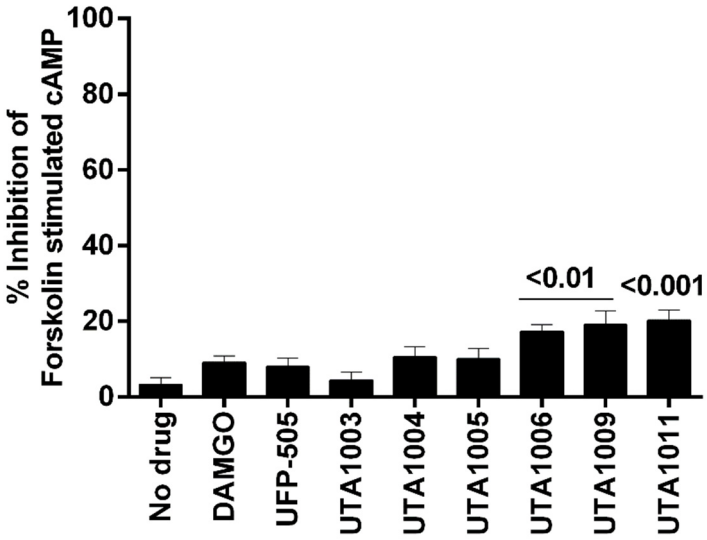


Figure 37. Opioid receptor specificity assessment of UTA-opioids using wild-type cells. Inhibition of forskolin-stimulated cAMP levels were measured in the absence ("no drug") or presence of 100µM UTA- and reference opioids. Statistically significant ($p < 0.05$) differences against the effect of 1 µM forskolin (no drug) are expressed as $p < 0.01$ or $p < 0.001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM (n = 6). Error bars are present in all graphs but are sometimes too small to be visible.

To investigate whether the novel UTA-opioids are MOP receptor agonists, all ligands were measured for their specificity in MOP receptor-expressing Chinese Hamster Ovary (CHO-MOP) cells (Fig. 38). All opioids significantly (one-way ANOVA, $F(7, 36) = 368$, $p < 0.0001$) inhibited forskolin-stimulated cAMP production (Fig. 38) and therefore, these ligands acted as MOP receptor agonists. Notably, all responses were comparable to those of the reference

compounds UFP-505 and DAMGO (Fig. 38).

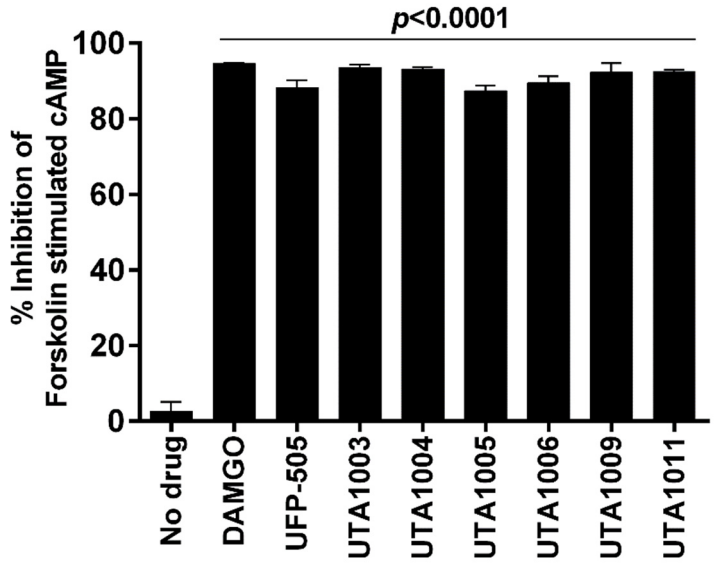


Figure 38. Effects of UTA-opioids on μ-opioid (MOP) receptor expressing cells. Inhibition of forskolin-stimulated cAMP levels were measured in the absence ("no drug") or presence of 100μM UTA- and reference opioids. Statistically significant ($p < 0.05$) differences against the effect of 1 μM forskolin (no drug) is expressed as $p < 0.0001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM (n = 6). Error bars are present in all graphs but are sometimes too small to be visible.

Since UFP-505 is described as a MOP receptor agonist / DOP receptor antagonist, I assessed whether the novel UTA-ligands showed any affinity towards the DOP receptor (Fig. 39). In this study, significant inhibition of forskolin-induced cAMP release was observed for all opioids. Both UFP-505 and UTA1003 showed significantly lower specificity than DPDPE (a DOP receptor agonist) (one-way ANOVA, $F(8, 32) = 52.09$, $p < 0.0001$) (Fig. 39). Beyond that, both ligands (UTA1003 and UFP-505) induced identical effects with regards to the DOP receptor. The remaining five UTA-opioids (UTA1004-006, UTA1009 and UTA1011) showed significant inhibition of forskolin-induced cAMP production (one-way ANOVA, $F(8, 32) = 52.09$, $p < 0.0001$), which was similar to the effects of DPDPE (Fig. 39).

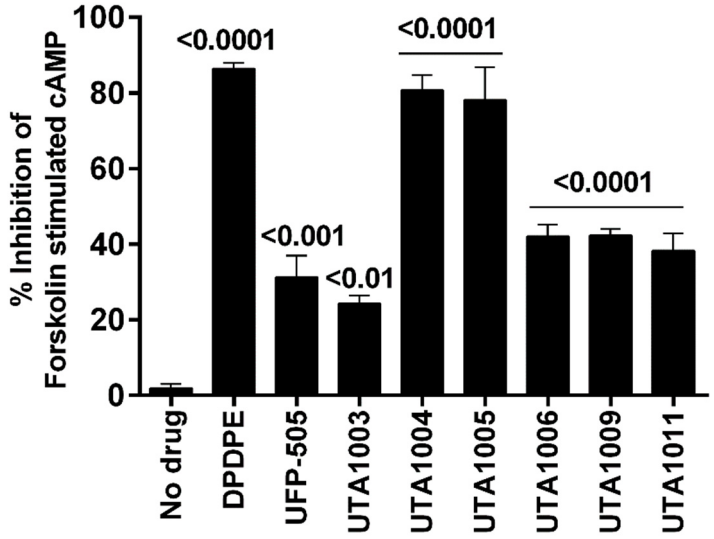


Figure 39. Effects of UTA-opioids on δ-opioid (DOP) receptor expressing cells. Inhibition of forskolin-stimulated cAMP levels were measured in the absence ("no drug") or presence of 100μM UTA- and reference opioids. Statistically significant ($p < 0.05$) differences against the effect of 1 μM forskolin (no drug) is expressed as $p < 0.01$, $p < 0.001$ or $p < 0.0001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

UFP-505 has been described as a partial agonist (pK_i 6.29) of the KOP receptor in a previous report (349). In this study, the specificity profile of UTA-opioids was measured using CHO-KOP cells with U50,488 (a KOP receptor agonist) as a reference compound (Fig. 40). Most ligands showed specificity for the KOP receptor except UTA1005 and UTA1006 (Fig. 40).

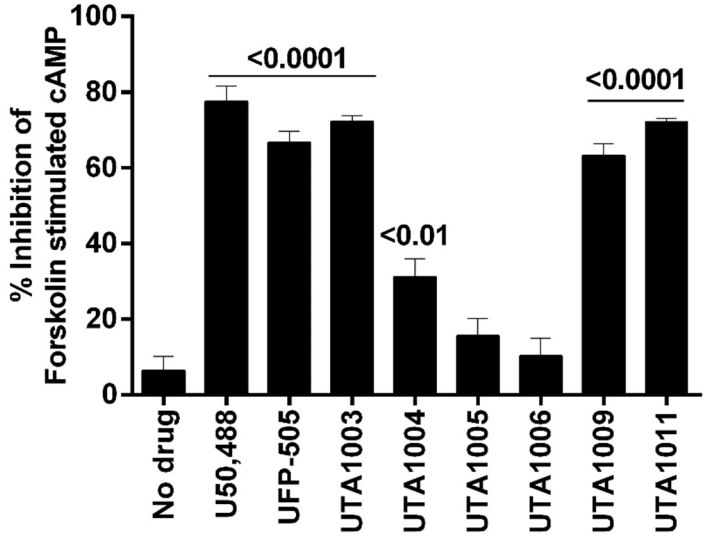


Figure 40. Effects of UTA-opioids on κ-opioid receptor expressing cells. Inhibition of forskolin-stimulated cAMP levels were measured in the absence ("no drug") or presence of 100μM UTA- and reference opioids. Statistically significant ($p < 0.05$) differences against the effect of 1 μM forskolin (no drug) are expressed as $p < 0.01$ or $p < 0.0001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

UFP-505 is known to be a partial agonist of the NOP receptor with a pK_i of 5.86 (349). In this study, all UTA-ligands were assessed for their effects on the non-opioid receptor (NOP receptor) using CHO-NOP cells (Fig. 41). In this experiment, most UTA-opioids except UTA1006 significantly inhibited forskolin-stimulated cAMP levels. However, their efficacy was significantly lower than the reference compound, nociceptin (a NOP receptor agonist) (one-way ANOVA, $F(8, 32) = 52.09$, $p < 0.0001$) (Fig. 41).

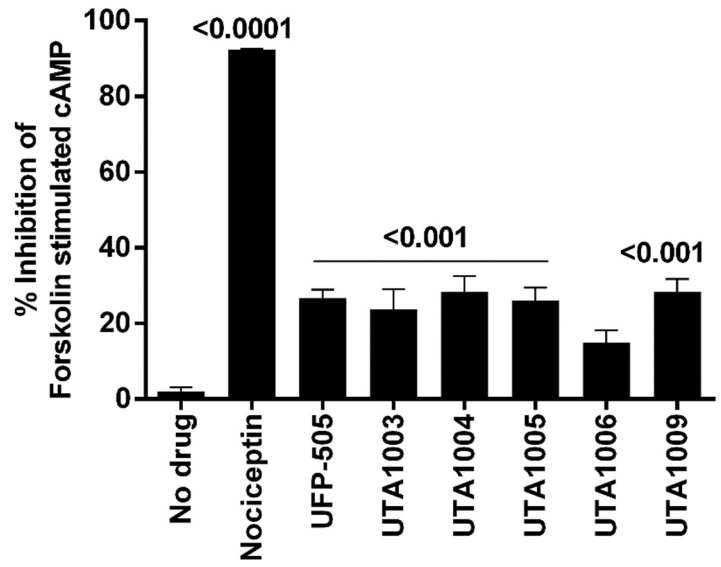


Figure 41. Effects of UTA-opioids on Non-opioid receptor expressing cells. Inhibition of forskolin-stimulated cAMP levels were measured in the absence ("no drug") or presence of 100μM UTA- and reference opioids. Statistically significant ($p < 0.05$) differences against the effect of 5 μM forskolin (no drug) are expressed as $p < 0.001$ or $p < 0.0001$ and were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM ($n = 6$). Error bars are sometimes too small to be visible.

6.3.3. Detailed agonistic effects of UTA1003 towards different opioid receptors

From the specificity profile assessment undertaken for this study, UTA1006, UTA1009 and UTA1011 are shown to be non-selective, as these ligands induced their effects on wild-type cells. In addition, UTA1004 and UTA1005 appeared to be full agonists of both MOP and DOP receptors. Based on the specificity profiles of UTA-1004, 1005, 1006, 1009 and 1011, it was concluded that these compounds were unlikely to provide achieve an improved adverse effect profile in comparison to opioids currently used in clinical practice for long-term treatment.

Therefore, UTA1003 was chosen as the most suitable ligand for further pharmacological characterisation, as it likely represented a MOP receptor agonist / DOP receptor partial agonist that promised the desired characteristics. Subsequently, EC_{50} values for UTA1003 and reference compounds (DAMGO, DPDPE, U50,488 and nociceptin) were determined from the dose-response curves for different opioid receptors.

In this study, the efficacy of UTA1003 for the MOP receptor was determined from a dose-response curve in CHO-MOP cells, where UTA1003 appeared to display similar effects to DAMGO, as their pEC_{50} and E_{max} values were indistinguishable (Fig. 42, Table 24).

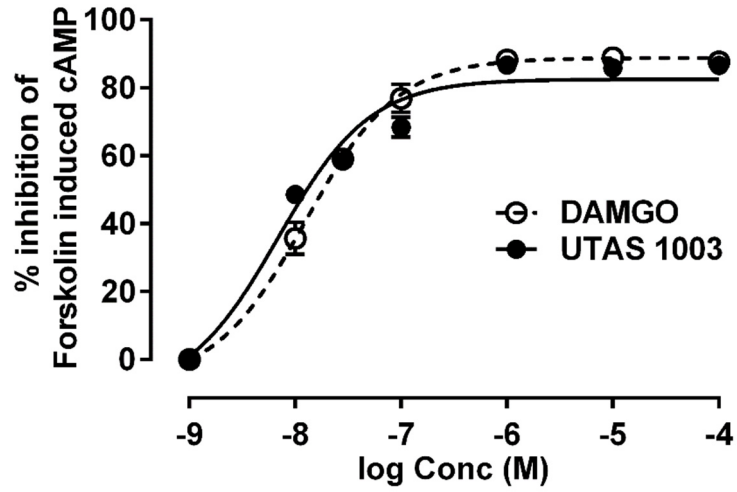


Figure 42. Dose-response of MOP-receptor agonistic effect of UTA1003. The MOP receptor agonistic effect of UTA1003 was assessed as inhibition (%) of forskolin-stimulated cAMP levels in CHO-cells expressing human MOP receptor. The cAMP was stimulated using 1µM forskolin and DAMGO (1 nM - 100 µM) was used as positive control. Values are represented as mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

Since UTA1003 displayed a similar effect to DAMGO in cells expressing the MOP receptor, I determined its efficacy (as EC_{50}) in cells expressing the DOP receptor (Fig. 43). A parallel dose-response curve and EC_{50} were determined for the selective DOP receptor agonist, DPDPE (Fig. 43). The pEC_{50} value of UTA1003 was similar to DPDPE (Table 24), but the magnitude of the efficacy (E_{max}) of UTA1003 was lower than the E_{max} of DPDPE (Fig. 43). Therefore, UTA1003 appears to be a partial agonist for the DOP receptor.

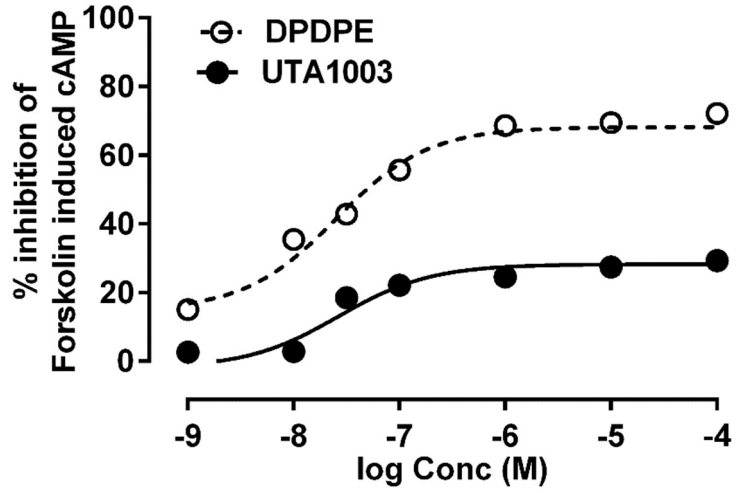


Figure 43. Dose-response of DOP-receptor agonistic effect of UTA1003. DOP receptor agonistic effect of UTA1003 was assessed as inhibition (%) of forskolin-stimulated cAMP levels in CHO-cells expressing human DOP receptor. The cAMP was stimulated using 5 µM forskolin and DPDPE (1 nM - 100 µM) was used as positive control. Values are represented as mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

During the screen with CHO-KOP cells, 100 µM UTA1003 induced a similar agonistic effect compared to U50,488 (Fig. 40). To investigate the magnitude of agonism of UTA1003 towards the KOP receptor, UTA1003 was further assessed in CHO-KOP cells (Fig. 44). Although UTA1003 at higher concentration showed similar efficacy to U50,488, the agonistic activity of this ligand is noticeably weaker than U50,488 (Fig. 44, Table 24).

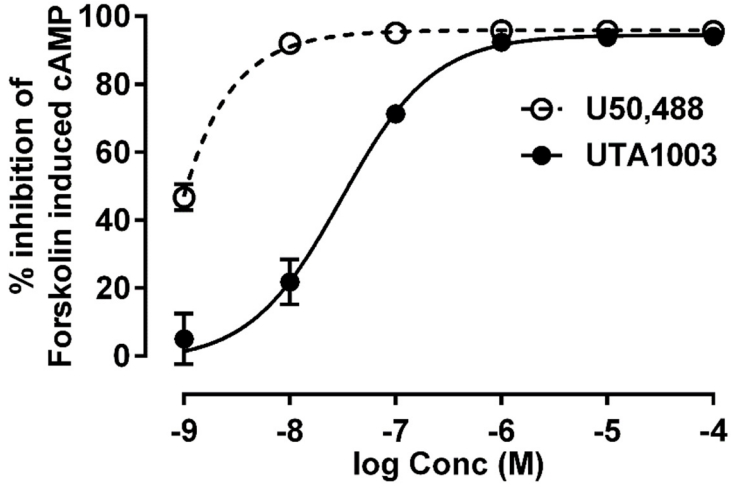


Figure 44. Dose-response of KOP-receptor agonistic effect of UTA1003. KOP receptor agonistic effect of UTA1003 was assessed as inhibition (%) of forskolin-stimulated cAMP levels in CHO-cells expressing human KOP receptor. The cAMP was stimulated using 5 µM forskolin and U50,488 (1 nM - 100 µM) was used as positive control. Values are represented as mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

Although UTA1003 showed significantly lower agonistic effects than nociceptin at high concentration (100 µM) (Fig. 41), I further investigated the agonistic effect of UTA1003 (0.1 nM to 100 µM) in CHO-NOP cells (Fig. 41). UTA1003 showed partial agonism (EC_{50} 1.79 µM) towards the NOP receptor. However, this effect was much lower compared to the results of the full agonist nociceptin (EC_{50} 0.69 nM) (Fig. 41, Table 24).

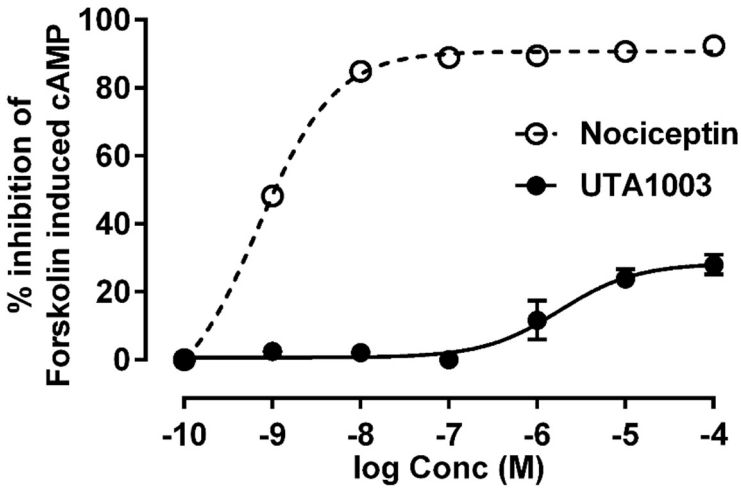


Figure 45. Dose-response of NOP-receptor agonistic effect of UTA1003. The NOP receptor agonistic effect of UTA1003 was assessed as inhibition (%) of forskolin-stimulated cAMP levels in CHO-cells expressing human NOP receptor. The cAMP was stimulated using 5 µM forskolin and nociceptin (0.1 nM - 100 µM) was used as positive control. Values are represented as mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

Table 24. Summary of receptor specificity and efficacy of UTA1003 for the major opioid receptors.

Cells	EC_{50} of UTA1003	pEC_{50} of UTA1003	Emax (%)	Ref. compound	EC_{50} of Ref. compound	pEC_{50} of reference	Emax (%)	Antagonist effect (K_d)
CHO-MOP	6.89 nM	8.16	82.44	DAMGO	12.5 nM	7.90	88.76	
CHO-DOP	26.6 nM	7.58	28.20	DPDPE	20.7 nM	7.68	68.18	47.8 nM
CHO-KOP	30.9 nM	7.51	94.47	U50,488	0.20 pM	12.70	95.94	
CHO-NOP	1.79 µM	5.75	28.34	Nociceptin	0.69 nM	9.16	90.73	

6.3.4. Antagonistic effect of UTA1003 on DOP receptors

A previous study found that some compounds that demonstrate low agonistic activity towards a particular receptor can also exhibit antagonistic effects (564). Since UTA1003 appeared to be a partial agonist (E_{max} 28.2 %) of the DOP receptor (Fig. 43), I further investigated its potential antagonism of this receptor using CHO-DOP cells (Fig. 46). The antagonist effect of UTA1003 was calculated as described in previous studies (160,349,560). The percentage (%) inhibition of forskolin-stimulated cAMP production was calculated for different concentrations of DPDPE with or without 2 μM UTA1003 in DOP receptor-expressing Chinese Hamster Ovary (CHO-DOP) cells (Fig. 46). Since the addition of UTA1003 (in DPDPE solution) shifted the dose-response curve of DPDPE (dose range: 10 nM to 10 μM) towards the right, expressed the characteristics of a DOP receptor antagonist (Fig. 46). The K_d value of UTA1003 was calculated as K_d = 47.8 nM using the formula, K_d = [UTA1003] / (CR-1), where CR is the ratio of the EC_{50} of DPDPE in the presence and absence of UTA1003 (160,306,560) (Fig. 46, Table 24).

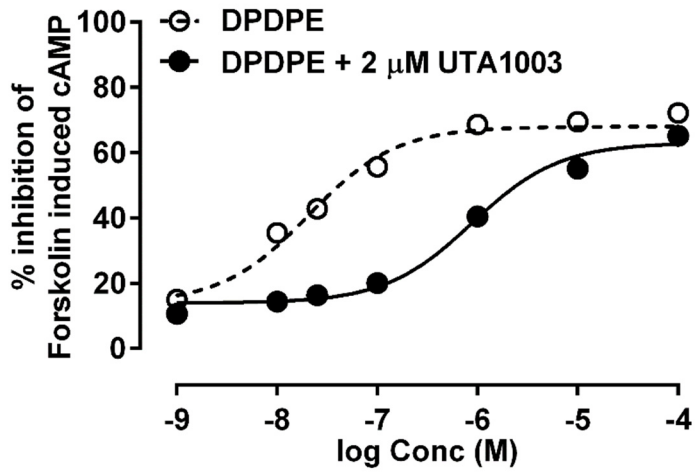


Figure 46. Antagonistic effect of UTA1003 against the effect of DPDPE on the δ-opioid receptor (DOP) expressing cells. Different molar concentrations (M) of DPDPE (1 nM - 100 μM) vs a combination of 2 μM UTA1003 and DPDPE used to assess the antagonism of UTA1003 in human DOP receptor expressing Chinese Hamster Ovary cells. The effects were assessed as inhibition (%) of 50 μM forskolin-stimulated cAMP levels. Values are represented as mean ± SEM (n = 6). Error bars are sometimes too small to be visualised.

6.4. Discussion

Several ligands with specificity on multiple receptors have been developed and characterised pre-clinically as this class of compounds promises to overcome some of the limitations of classical opioid ligands during long-term treatment in the clinic (150,155,160,337). Importantly, several compounds based on "Dmt-tic"-containing peptides have also been described to display MOP receptor agonist / DOP receptor antagonist profiles (348,351,352,353). UFP-505 is a peptide based on 'Dmt-Tic' structure that was characterised *in vitro* and *in vivo*. Currently, it is thought that the "Dmt-Tic" moiety of the peptide ligand is required for DOP receptor antagonism, while the Bzl (Benzylamine) and spacer (Gly-NH) moieties are required for MOP receptor agonism (347,348). Noticeably, UFP-505 induced antinociception with a reduced antinociceptive tolerance after intrathecal injections but did not show efficacy after systemic or oral administrations (354). In the current study, I tested structural analogues of UFP-505 to gain more information about a structure-activity relationship of this class of compounds. This project was initiated to evaluate the possibility to develop an analogue of UFP-505 that shows antinociception after systemic administration.

In this study, I measured the functional activities of the structural analogues of UFP-505 (UTA-ligands) by measuring their effects on cellular cAMP levels in a similar way to previous studies (178,560). the cAMP assay is a high-throughput and sensitive assay for screening G-protein coupled receptor (e.g. opioid receptors) mediated physiological pathways (565). This assay is user-friendly, as it does not require radioactive materials, which are required for classical radio-ligand binding and [^{35}S]GTPγS assays (565,566). However, a compound like a forskolin is needed to increase the production of cellular cAMP levels by stimulating adenylyl cyclase, as basal cellular cAMP levels are typically low (567). A previous study found that cAMP assay produced similar specificity profile of opioids to the receptor binding assay, but it produced

better resolution than [^{35}S]GTPγS assay (560). Therefore, an evaluation of downstream signalling (e.g. cAMP assay) might be comparatively a better tool than [35S]GTPγS assay to measure the specificity profile of an opioid.

In this study, UFP-505 appeared to be a MOP receptor agonist / DOP receptor partial agonist as previously reported (348,349,353). However, these results are in conflict with another study that characterised UFP-505 as a full agonist of the DOP receptor with partial agonism for MOP and KOP receptors (560). Surprisingly, this study found no significant efficacy of UFP-505 (maximum stimulation < 10 %) for all major opioid receptors (MOP, DOP and KOP) using a [^{35}S]GTPγS binding assay (560). In the same study, morphine and endomorphine only produced about 50 % effects for the MOP receptor (560), indicative of a low sensitivity of the employed [35S]GTPγS binding assay. Thus, the differential pharmacological profiles of UFP-505 reported by previous studies may be a consequence of variations in assay methods and the experimental settings used by different laboratories. On the other hand, the results of this study agree with my results in that UFP-505 showed partial DOP receptor agonism with a 28 % inhibition of forskolin-induced cAMP stimulation (560), which is very similar to my result (31 % inhibition).

The pharmacological profile of UFP-505 is not fully clear in the existing literature. UFP-505 was initially described in the literature as a DOP antagonist by Dietis and colleagues, having a strong affinity for DOP receptors (pK_i 9.82 in radio-ligand displacement assays) with a non-significant EC_{50}, but a significant antagonistic potency of pK_b 9.81 in a GTPγ^{35}S assay and a pA_2 9.15 in the electrically stimulated guinea pig ileum, both against DPDPE (349). However, in a recent study published by Dietis and co-authors (350), looking at UFP-505's characterisation in more depth, the ligand was shown to produce a weak partial agonism in cell

systems with very high DOP-receptor expression (E_{max} approximately 7% normalized for DPDPE E_{max} 100% in a GTPγ^{35}S assay; E_{max} approximately 18% with DPDPE at E_{max} 78% in forskolin-stimulated cAMP inhibition assay). The authors (Dietis et al.) concluded that UFP-505 "displays a variable expression-dependent efficacy at the DOP receptor" (350). This phenomenon has been previously described for other ligands (559). These data agree with the findings in my thesis (Fig. 39) and provide an explanation of the characterisation of UFP-505, which we now know that behaves as a DOP antagonist in systems with receptor expression closer to that of primary tissues, but as low efficacy partial agonist in systems with very high DOP receptor expression levels.

The replacement of a benzene ring from the "Tic" peptide of UFP-505 in this study produced UTA1003 that subjectively displayed better solubility in aqueous solution, in line with a significantly reduced logP value (-0.11 versus 0.41), although I did not perform detailed solubility experiments. At the same time, this structural change resulted in comparable MOP receptor agonism with slightly reduced DOP receptor antagonism (pK_b: 7.32) on compared to UFP-505 (pK_b: 9.81(349); 10.50 (348)). The dose-dependent efficacy of UTA1003 towards the MOP receptor was similar to DAMGO, but its efficacy towards DOP and NOP receptors was substantially lower than the specific reference compounds DPDPE and nociceptin. On the other hand, at high ($\geq$ 1 μM) concentrations UTA1003 acted as an agonist of the KOP receptor similar to U50,488, which was noticeably lower than U50,488 at low concentrations (< 1 μM). The detailed dose-response analysis of UTA1003 indicates that UTA1003 is also a KOP receptor agonist / NOP receptor partial agonist, which is similar to previous reports regarding UFP-505 (349). UFP-505 was described to have affinity at low μM range (pKi 6.26 in CHO-KOP cells) using radio-ligand binding assay and a non-significant efficacy using the GTPγ^{35}S assay (349). Therefore, deletion of the benzene ring from "Tic" pharmacophore in UTA1003 appears to

have only a minor impact on the pharmacological efficacy of "Dmt-Tic" peptides apart from increased efficacy towards KOP receptor.

Previous studies hypothesised that the "Gly-NH-Bnz" peptide moiety is essential for the MOP receptor agonistic activity of peptide opioid ligands (347,348,349). However, in contrast to this hypothesis, the replacement of glycine (Gly-NH) with a hydrocarbon chain in UTA1009 and UTA1011 retained their agonistic activity towards the MOP receptor. Since this structural change also generated non-selective compounds that acted on both opioid and non-opioid receptors, the "Gly-NH" moiety appears to be important to maintain opioid receptor specificity overall. However, one possibility to reconcile the conflicting data could be that instead of the Gly-NH motive, the benzyl (Bnz) group could be responsible for the MOP receptor specificity. However, although I did not investigate this hypothesis, due to restrictions with regards to the number of UFP-505 analogues that could be synthesised.

In this chapter, the roles of "Dmt-Tic" and "Gly-NH" peptide moieties for the pharmacological profile of different UFP-505 analogues are described. I also identified a novel opioid ligand (UTA1003) with MOP and KOP receptor agonist and DOP receptor partial agonist profile (i.e. mixed activity on multiple opioid receptors). The specificity of the UTA-compounds was determined by measuring their efficacy on different types of opioid receptors, which could be measured as selectivity in a radio-ligand binding assay. This is a limitation of my study, which can be addressed in future studies. In this study, single concentration of UTA-opioids (100 μM) were used to determine the specificity of the ligands, which is probably difficult to draw the conclusion on the pharmacological profile of every ligand. The study provided substantial information to screen the ligands to select the best compound for the preclinical study (chapter-7). The cAMP assay is now widely used to characterise opioid ligands (160,178,560) and

therefore the inference from the results is valid. I also agree that full concentration curves of every ligand would provide a better basis for my conclusion. This experimental approach could not be completed due to the associated cost and time-restrictions for my work. I used the cAMP assay to choose the best ligand by determining its specificity profile of through screening by using a single concentration of all ligands and then chose the best ligand (UTA1003) for the full concentration curves of its agonism on all types of opioid receptors and antagonism for DOP receptor. The selectivity profile of UTA1003 is complete as its efficacy is measured using 6-7 concentrations of UTA1003 and compared with reference opioids selective for a particular opioid receptor (e.g. DAMGO, DPDPE). I conducted this study in a way to reduce the cost without affecting ultimate objective of the research project. I can not exclude the possibility of an antagonistic effect of another ligand such as UTA1006 on the DOP or KOP receptors. A detailed screening with multiple concentrations could investigate more UTA-ligands with mixed activity on multiple receptors in the future. Nevertheless, these experimental results were the prerequisite for a preclinical *in vivo* study with UTA1003 to assess its effects on antinociception, antinociceptive tolerance and motor behavioural effects using a repeated treatment regimen.

CHAPTER SEVEN

Differential effects of UTA1003 on antinociceptive tolerance and behaviour

Preface to chapter seven

The novel opioid ligand UTA1003 was described in *chapter six* and was selected as the most promising ligand due to its mixed activity on multiple opioid receptors. In this study (*chapter seven*), I investigated the role of UTA1003 on antinociception and behaviour after acute treatment compared to morphine. Furthermore, repeated treatment of UTA1003 and morphine over a period of eight days was carried out to observe the role of UTA1003 on the development of antinociceptive tolerance and behavioural effects.

7. Differential effects of the UTA1003 on antinociceptive tolerance and behaviour

Abstract

Analgesic tolerance is a major problem in the clinic for the maintenance of opioid-induced long-term pain-relief. Opioids with mixed activity on multiple opioid receptors promise reduced antinociceptive tolerance in preclinical studies, but these compounds typically have poor bioavailability upon oral, subcutaneous, intraperitoneal or intravenous administration. UTA1003 is a novel opioid that acts as MOP and KOP receptor agonist and as a partial agonist for the DOP receptor. In the present study, its antinociceptive effects, as well as its effects on antinociceptive tolerance and motor behaviour were investigated in male rats. Acute antinociception was measured before (basal) and at different time-points after subcutaneous injection of UTA1003 and/or morphine using tail-flick and hot-plate assays. Different motor behaviours, including horizontal locomotion, rearing and turning were automatically measured in an open-field arena. The repeated twice-daily dosage of UTA1003 and morphine was also conducted over a period of eight days. In these experiments, UTA1003 induced mild antinociceptive effects after acute administration but induced no tolerance after repeated treatment. UTA1003, as a co-treatment with morphine also prevented morphine tolerance. UTA1003 showed less motor suppression than morphine in both acute and sub-chronic treatment regimens, while it did not affect morphine-induced motor suppression or hyper-excitation. Based on these activities, I speculate that UTA1003 crosses the blood-brain barrier after subcutaneous administration and therefore could be developed as a lead molecule to avoid opioid-induced antinociceptive tolerance and motor suppression. However, further structural modifications to improve its antinociceptive effects, its toxicity profile and ADME parameters are nevertheless required.

Keywords: UTA1003; UFP-505; morphine; antinociception; antinociceptive tolerance; motor behaviour; mixed opioid; mixed activity.

7.1. Introduction

Opioids, especially morphine and other MOP receptor agonists, are widely used drugs for the clinical treatment of chronic pain, (22,568,569). However, long-term repeated use of clinical opioids such as morphine is often limited by their liabilities to induce a significant range of side-effects, such as respiratory depression, sedation, constipation, addiction, dependence, withdrawal symptoms, behavioural suppression and analgesic tolerance (111,112). Tolerance is a phenomenon of reduced efficacy of an effect of a drug over repeated use of particular dose (113). Previous studies also showed that there are differences in onset or magnitude of tolerance to antinociceptive and other behavioural effects, such as sedation, nausea, ventilatory or respiratory depression (172,570). For example, antinociceptive tolerance to morphine did not show tolerance to its ventilatory depressant effects in the rat (571). Similarly, tolerance to respiratory depression is relatively slower than tolerance to euphoric effect (572). On the other hand, tolerance to constipation develops slower than respiratory depression, sedation or nausea (573,574).

Antinociceptive effects and antinociceptive tolerance of morphine are both dependent on dose, dosing frequency and duration of treatment (137). In contrast, morphine-induced behavioural side-effects in the clinic are both reported as dose-dependent (e.g. pruritus) or dose-independent (e.g. nausea, vomiting) (250,251,440). At present, the collective evidence suggests that antinociceptive tolerance to morphine does not necessarily translate to tolerance against behavioural side-effects.

Therefore, many studies investigated different ways to prevent antinociceptive tolerance to morphine. Early studies showed that repeated administration of a combination of morphine (a MOP receptor agonist) and naltrindole or another DOP receptor antagonist showed better

antinociception and less antinociceptive tolerance compared to morphine alone (122,123,124,125). To achieve this, a significant effort was made to identify single ligands that are non-selective or have mixed activity on multiple opioid receptors, as these compounds promise better pharmacokinetic/pharmacodynamic profiles than a combination of drugs (280,282,346). As a result, MOP and DOP receptor selective opioid ligands with mixed activity have been described that indicated reduced antinociceptive tolerance compared to clinically used opioids (150,151,155,160,178,184,337). Especially, MOP receptor agonist / DOP receptor antagonist ligands showed less antinociceptive tolerance compared to morphine or fentanyl (150,155,160,337,346). A few of these MOP receptor agonist / DOP receptor antagonist ligands showed less physical dependence or reward-responses compared to clinically used opioids (337,346). Similarly, some MOP / DOP receptor agonists also reduce antinociceptive tolerance (151,171,178,184), which indicates MOP and DOP receptor interactions are essential to manage antinociceptive tolerance, although the detailed mechanisms underlying this effect are not understood. On the other hand, MOP / KOP receptor agonists reduce cocaine abuse after co-administration (280,575), while MOP / NOP agonist ligands have been reported as non-addictive analgesics and are effective to treat neuropathic pain (283,308). However, repeated administration of the MOP / NOP receptor agonist ligand BU08028 induced earlier antinociceptive tolerance compared to morphine, (152), which illustrates that the entire activity profile of all novel bi-functional ligands has to be determined to be in a position to predict their clinical usefulness. Noticeably, for many new opioids, their antinociceptive tolerance induction after repeated treatment (see chapter one) has not been reported, which significantly hinders the detailed interpretation of experimental data and their clinical development.

UTA1003 is an opioid ligand recently developed at the University of Tasmania, Australia that showed a pharmacological profile as MOP and KOP receptor agonist and displayed variable effects (partial agonist and antagonist) towards the DOP receptor. UTA1003 is based on the previously described bi-functional opioid UFP-505 and shows similar activity (EC_{50} 6.89 nM) as the standard MOP receptor agonist DAMGO (EC_{50} 12.5 nM), while also antagonising the DOP receptor (pK_b 7.32) (unpublished data, see chapter six). In a recent study, UFP-505 is also reported as a partial agonist of DOP receptor shown to produce a weak partial agonism in cell systems with very high DOP-receptor expression (350). In this study, Dietis and colleagues concluded that UFP-505 "displays a variable expression-dependent efficacy at the DOP receptor" (350). A similar phenomenon has been described previously for other ligands (559). I hypothesised that the UTA1003 may induce less antinociceptive tolerance and behavioural side-effects than morphine, as UTA1003 is a MOP receptor agonist / DOP receptor partial agonist. In addition, I speculated that after co-administration with morphine over a period of several days, UTA1003 might also reduce the antinociceptive tolerance and behavioural side-effects of morphine. Therefore, the current study investigated the motor behavioural side-effects, antinociception and antinociceptive tolerance after repeated administration of UTA1003, morphine and their combination. This study, for the first time, describes that the novel ligand UTA1003 is a potential drug-candidate to induce antinociception with reduced adverse effects compared to morphine.

7.2. Materials and methods

7.2.1. Materials

Dimethyl sulfoxide (DMSO) and sodium chloride were purchased from Sigma-Aldrich. Morphine sulphate was obtained as 30 mg/ml stock solution (Hameln Pharmaceuticals GmbH, Germany), while UTA1003 and the reference compound UFP-505 were synthesised by A/Prof Jason Smith (School of Chemistry, University of Tasmania). Stock solutions and final dilutions of these drugs were prepared under aseptic conditions using 10 % DMSO in 0.9 % sodium chloride solution as a vehicle.

7.2.2. Animal maintenance and care

Thirty male Sprague-Dawley (SD) rats (253.6 ± 3.9 g, 8 week old), obtained from the University of Tasmania animal services were housed as three littermates per cage at 22 ºC with 50-60 % humidity under an automated 12-hour day/night cycle (lights on at 7:00 am) with free access to food (Barastoc rodent cubes, Ridley Corporation, Melbourne, Australia) and water. Only male rats were used to avoid hormonal effects in female rats (336). All procedures and handling were approved by the University of Tasmania Animal Ethics Committee (A0013864) and were conducted according to *The Australian Code for the Care and Use of Animals for Scientific Purposes* (434). The experiments were conducted in compliance with the ARRIVE guidelines (435).

7.2.3. Treatment protocol

Animal body weights were recorded daily, immediately prior to experiments in order to determine the dosage for each rat. The dosage of UFP-505, UTA1003 and morphine were calculated based on individual body weights. All drugs were administered as daily subcutaneous injections between the left thigh and the spinal cord. All rats were randomly

divided as previously described (448) into five subgroups (n = 6, each) (morphine 3 mg/kg b.i.d.; UFP-505 27.1 mg/kg acute administration; UTA1003 24.6 mg/kg b.i.d.; a combination of morphine (3 mg/kg b.i.d.) and UTA1003 (24.6 mg/kg b.i.d.); or vehicle (DMSO 10% in 0.9% sodium chloride solution, acute administration). Opioids were administered twice daily (mornings and evenings) over a period of 8 days (except for UFP-505 and vehicle). Vehicle and UFP-505 were excluded from long-term testing due to their negligible antinociceptive *in vivo* effects on day 1. UFP-505 and UTA1003 doses were calculated to provide the same molar concentrations compared to 40 mg/kg morphine sulphate. The amount of injected DMSO was kept equal for all used solutions. Reduced laboratory illumination intensity prior and during experiments minimised any potential discomfort to the animals.

7.2.4. Assessment of antinociception

Nociceptive thresholds were determined using two independent assays (tail-flick & hot plate) using commercially available equipment (Ugo Basile, Comerio, Italy). Maximum exposure to the nociceptive thermal stimulus was 15 sec for the tail-flick and 30 sec for the hot-plate assay. The infrared intensity of the tail-flick photocell was set to 30, whereas the temperature of the hot-plate was set to 54 ±0.5 °C. On the first treatment day, all animals were tested in both assays immediately prior to the vehicle or opioid administration to obtain basal measurements as well as values for 15, 30, 60 and 120 min post-administration. On all other days, the rats were tested prior- and 15, 30 min post-injection. Nociception measurements were conducted in a blinded manner and the mean of three independent measurements for each time-point with a 1 min interval between measurements was recorded to minimise the 'handling' effects. The maximum possible effect (MPE) was defined as MPE % or antinociception = 100 X [(test latency – baseline latency)/(cut-off time – baseline latency)] as previously described (436). The area under the curves (AUC) were calculated by the trapezoid method using GraphPad Prism V6

software (GraphPad Software Inc., La Jolla, CA, USA).

7.2.5. Behavioural Measurements

Behaviour was tested in the open-field arena of a fully automated Multi-Conditioning System (MCS, TSE GmbH, Homburg, Germany) that is able to assess and simultaneously analyse a large range of behavioural parameters of animals kept under controlled conditions. The MCS platform included an internal noise/light/temperature insulation system and a 3D infrared-beam frame that provided fast and accurate animal movement detection (100Hz), combined with a high-resolution video monitoring and automated movement tracking system. Quantification and visualisation of the MCS data were processed by integrated system software (TSE ActiMot). The open-field arena was fully cleaned and dried after testing of each animal. A background white noise (10 dB) generator was used during all experiments in order to cancel out any unexpected laboratory sounds. On the 1st treatment day, behaviour was assessed 1 min after nociception testing at all time-points over a period of 5 min, while the rats were tested only 30 min post-injection on the subsequent treatment days. Behavioural testing for this study included six different activity parameters (moving time, total distance travelled, rotation numbers, rearing numbers, rotation time and rearing time). Rotation numbers were summarised from clockwise and counter-clockwise rotations as detected by the MCS. The area under the curves (AUC) for behavioural parameters were calculated by multiplication of behavioural effects (e.g. moving time) and treatment period (min or day). At the end of the observation period, animals were anesthetised with 5% (w/v) isoflurane in oxygen at a flow rate of 1 L/min, until the animal was unconscious (usually 5-7 min), before decapitated.

7.2.6. Statistical analysis

Data are expressed as mean ±SEM and analysed by one-way ANOVA with Dunnett's multiple comparisons test or unpaired t-test, using GraphPad Prism V6 software (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparison (Dunnett's or Sidak's test) was employed when F achieved $p < 0.05$ and there was no significant variance in homogeneity. A 'p' value less than 0.05 was considered statistically significant.

7.3. Results

7.3.1. Toxicity of UTA1003

In the present study, I did not observe any physiological or behavioural toxicity in our animals after subcutaneous administration of UTA1003 and UFP-505. Repeated morphine dosing is known to induce itching (253,254,576), which was absent in the UTA1003-treated animals in this study. This is an important advancement as a previous study reported behavioural toxicity for two MOP / NOP receptor agonists after subcutaneous administration (283).

7.3.2. Antinociceptive effect of UTA1003 after acute administration

Two opioids with mixed selectivity on different opioid receptors, UFP-505 and UTA1003 were evaluated for their antinociceptive effects after acute sub-cutaneous injections using male Sprague Dawley rats on day 1. Their antinociceptive effects were compared to morphine or vehicle on day 1. Before opioid administration, no significant differences in nociceptive levels were observed in the five different treatment groups using both the tail-flick and hot-plate antinociception tests (Fig. 47). It indicates that none of the animals used in this study demonstrated hyperalgesia or increased pain-sensitivity. Baseline antinociception measurement is very important from a clinical perspective, as people experience hyperalgesia after long-term treatment with low dose opioids (577,578). Hyperalgesia has also been observed in preclinical studies, although the exact mechanism by which it occurs is not completely understood (180,579). Previous studies show that hyperalgesia is not mediated by the brain MOP receptor and is also not associated with plasma concentration of morphine-3-glucuronide, but rather a consequence of protein kinase C gamma (PKCγ) and NMDA receptor subtype NR1 upregulation in the spinal cord (257,258). Vehicle-treated animals did not show any antinociceptive effects or hypersensitivity in both antinociception tests over a period of 120 min after administration (Fig. 47 A, B). However, 15 min post administration, morphine-

induced significant antinociception compared to vehicle-treated animals (unpaired t-test; $t(6) = 17.49$; $p < 0.0001$) before it decreased gradually until 120 min (unpaired t-test; $t(8) = 5.96$; $p < 0.001$) (Fig. 47 A, B). UTA1003 induced significant antinociception already 15 min post-injection in the hot-plate assay (unpaired t-test; $t(9) = 2.53$; $p < 0.05$) and from 30 min onwards in both assays (unpaired t-test; $t(5) = 4.544$; $p < 0.01$ (tail-flick); $t(7) = 3.35$; $p < 0.05$ (hot-plate)) (Fig. 47 A, B). The antinociceptive effect of UTA1003 was significantly different from the effects of vehicle until 60 min (unpaired t-test; $t(9) = 4.08$; $p < 0.01$) using hot plate (Fig. 47 B) and 120 min (unpaired t-test; $t(9) = 2.483$; $p < 0.05$) using tail-flick assay (Fig. 47 A) respectively.

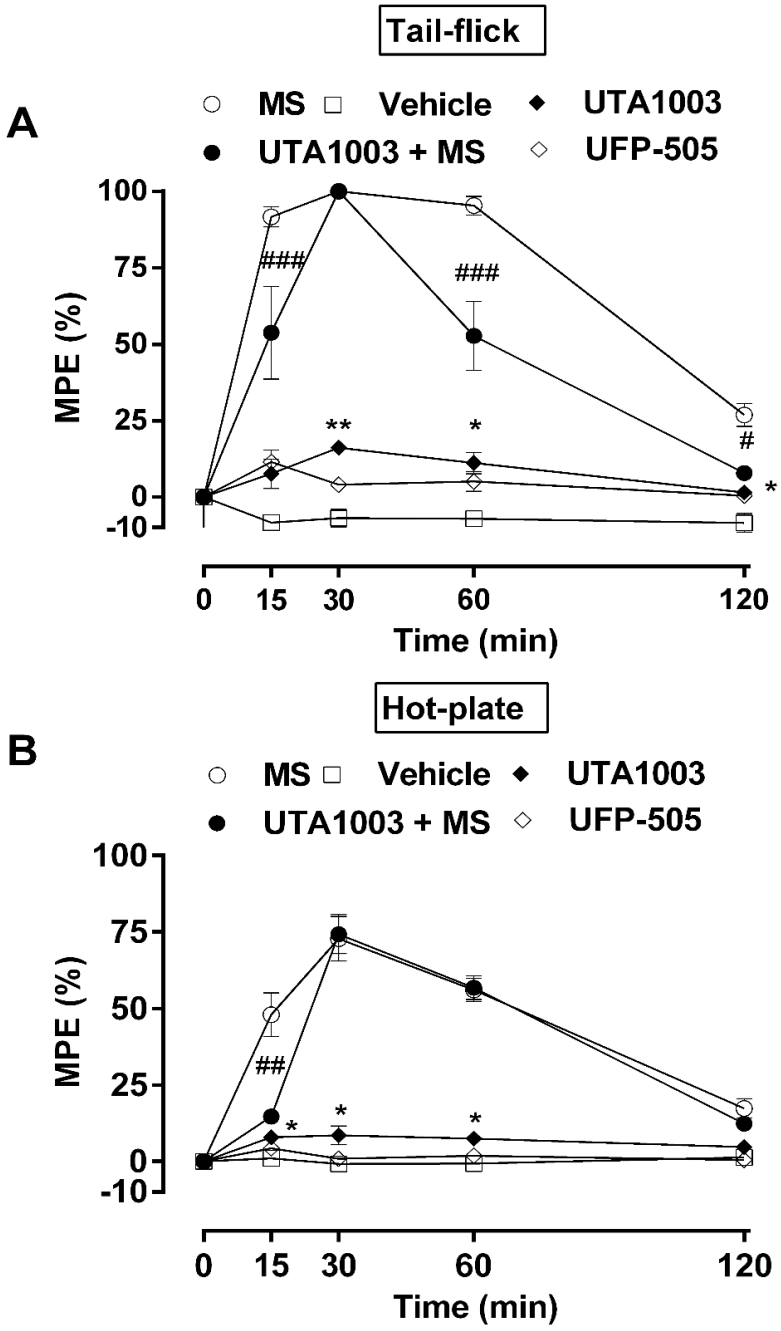


Figure 47. Antinociceptive effects of different opioids after acute treatment. Antinociceptive effects after single subcutaneous injections of UFP-505 (27.1 mg/kg), UTA1003 (24.6 mg/kg), Morphine (MS, 3.0 mg/kg) or vehicle were measured in Sprague Dawley rats. Antinociception was measured over a period of 120 min using tail flick (A) and hot plate (B) assays. Statistical significance against the effect of vehicle is shown as *$p < 0.05$ or **$p < 0.01$ for the same time-point was calculated using unpaired t-test. Additionally, statistically significant differences between MS and MS+UTA1003 treated animals for the same time-point was calculated using unpaired t-test and are shown as $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$. Values are presented as Mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

The combination of UTA1003 and morphine-induced significant antinociception from 15 min post-administration (unpaired t-test; $t(4) = 4.30$; $p < 0.05$) in the tail-flick assay while antinociception peaked at 30 min in both antinociceptive assays (unpaired t-test; $t(7) = 29.44$; $p < 0.0001$ (tail-flick); $t(10) = 11.47$; $p < 0.0001$) (Fig. 47 A, B). Subsequently, the antinociceptive effect of this drug combination gradually declined over the 120 min observation period (unpaired t-test; $t(10) = 4.25$; $p < 0.01$) (Fig. 47 A, B). However, the antinociceptive effect of the UTA1003 / morphine combination produced significantly less antinociception than morphine alone at 15 min (unpaired t-test; $t(6) = 7.25$; $p < 0.001$ (tail-flick); $t(9) = 4.16$; $p < 0.01$) and 60 min (unpaired t-test; $t(7) = 6.55$; $p < 0.001$; tail-flick assay) post-administration (Fig. 47 A, B). In comparison, over the 2 hour observation period UFP-505 only showed some minor but statistically significant antinociception at 15 min post-injection (unpaired t-test; $t(7) = 3.18$; $p < 0.05$) (Fig. 47 A, B).

7.3.3. Effect of UTA1003 on morphine-induced antinociceptive tolerance

To investigate the effects of UTA1003 on antinociceptive tolerance and its interaction with morphine, opioids were administered individually or in combination twice daily over a period of 8 days. Antinociceptive effects of these opioids were measured daily using tail-flick and hot plate tests 30 min post-administration. Antinociceptive effects of UTA1003 were 27 ± 3.73 % and 22.57 ± 1.77 % MPE respectively in the tail-flick and hot-plate assays respectively (Fig. 48 A, B). However, there was no significant reduction (tolerance) or increment (hyperalgesia) of antinociception observed in this group of animals over the course of the 8 day observation period (Fig. 48 A, B). Therefore, UTA1003-induced antinociception (% MPE) on day 8 was 20.52 ± 2.13 % and 15.47 ± 2.55 % respectively for tail-flick and hot-plate assays (Fig. 48 A, B).

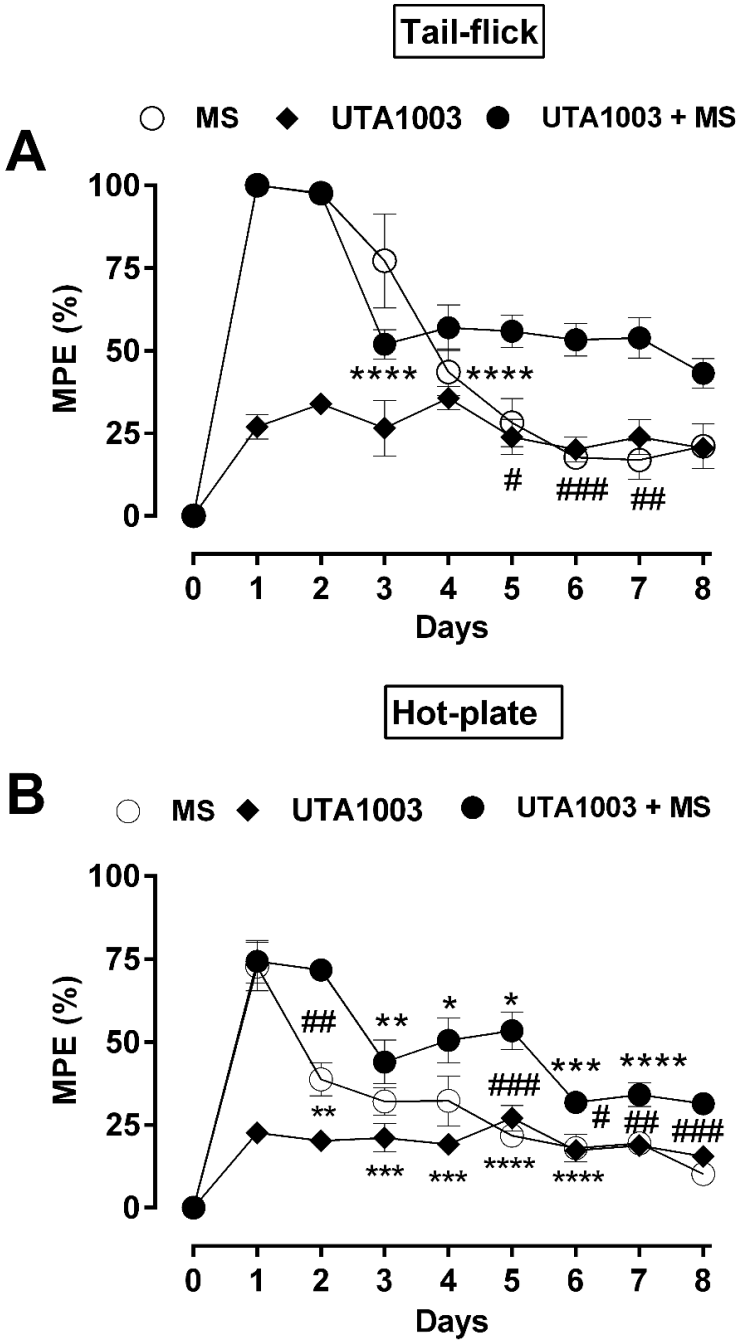


Figure 48. Antinociceptive effects of chronic administration of opioids. Antinociceptive effects after twice-daily subcutaneous treatment with UTA1003 (24.6 mg/kg), morphine (MS, 3 mg/kg) or their combination were measured in Sprague Dawley rats. Antinociception was measured daily at 30 min post injections over a period of 8 days using tail flick (A) and hot plate (B) assays. Statistical significance ($p<0.05$) against the effect of day-0 was calculated using One-way ANOVA and is shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$. Additionally, statistically significant differences between MS and MS+UTA1003 treated animals for the same time-point was calculated using unpaired t-test and are shown as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$. Values are presented as Mean ± SEM (n = 6). Error bars are sometimes too small to be visible.

On the other hand, morphine produced complete antinociception (100 % MPE) on day 1 in the tail-flick assay. The combined treatment of UTA1003 and morphine also showed similar antinociception levels comparable to morphine itself on day 1 (Fig. 48 A, B). The full antinociceptive effects of morphine were maintained until day 2 and then a statistically significant reduction was observed on day 4 (one-way ANOVA; $F(7, 27) = 23.33$; $p < 0.0001$) using tail-flick assay (Fig. 48 A). Beyond that, morphine-induced antinociception gradually declined over the observation period (Fig. 48 A). In contrast, the UTA1003 / morphine-treated animals showed tolerance from day 3 (one-way ANOVA; $F(7, 29) = 25.20$; $p < 0.0001$) and the effect was (51.94 ± 4.46 %) (Fig. 48 A). These co-treated animals maintained the anti-nociceptive effects of the drug combination until day 8 (43.17 ± 4.45 %), with no statistical differences between days 3 and 8 (Fig. 48 A). Noticeably, the combination of UTA1003 / morphine-induced significantly higher antinociception levels from days 5 (unpaired t-test; $t(8) = 3.16$; $p < 0.05$) to 7 (unpaired t-test; $t(6) = 4.36$; $p < 0.01$), compared to morphine-treated animals (Fig. 48 A).

The antinociception of morphine and the combination of UTA1003 / morphine were further investigated using the hot-plate assay where both groups showed similar antinociception on day 1 (72.77 ± 7.30 % and 74.28 ± 6.41 % MPE respectively) (Fig. 48 B). Morphine-treated animals showed antinociceptive tolerance from day 2 (one-way ANOVA; $F(7, 29) = 16.02$; $p < 0.01$), which decreased gradually over time until day 8 (Fig. 48 B). UTA1003 did not show signs of antinociceptive tolerance until day 3 (one-way ANOVA; $F(8, 37) = 20.41$; $p < 0.01$). Beyond that, a 50% antinociceptive effect was maintained until day 5 (Fig. 48 B). This effect further decreased over time until the end of my observation period (Fig. 48 B). Noticeably, the antinociceptive effects of morphine were significantly lower than those of the UTA1003 / morphine co-treatment on day 2 (unpaired t-test; $t(5) = 5.38$; $p < 0.01$) and from day 5 (unpaired

t-test; t (10) = 5.02; $p < 0.001$) to day 8 (unpaired t-test; t (6) = 6.15; $p < 0.001$) (Fig. 48 B).

7.3.4. Effect of acute administration of UTA1003 on motor behaviour

To gain a basic understanding of the behavioural effects of UTA1003, motor behaviour was analysed using an open-field paradigm over a period of 120 min after drug administration. These effects were compared to vehicle- and morphine-treated animals. The behavioural parameters assessed in the open-field arena were divided into three major categories: locomotion, rotation and rearing.

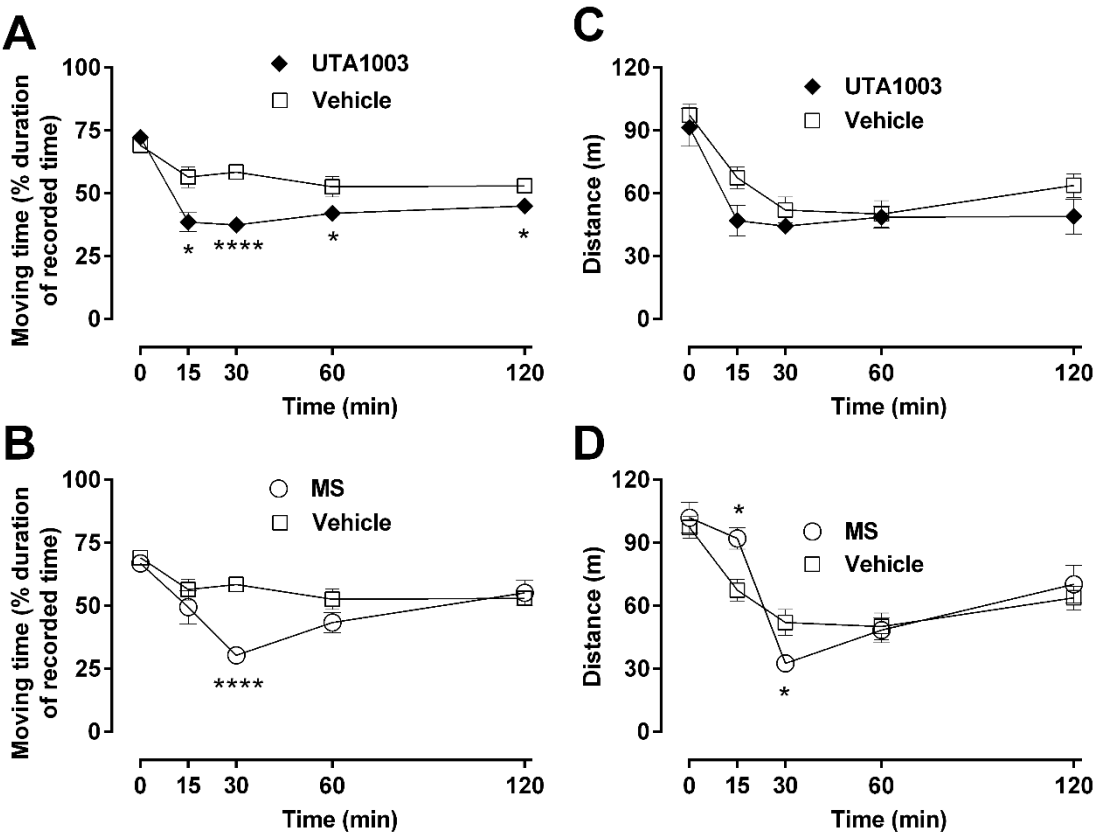


Figure 49. Acute time-dependent effects of morphine and UTA1003 on locomotor activities. Open-field locomotor activities after acute subcutaneous injections of UTA1003 (24.6 mg/kg), morphine (MS, 3.0 mg/kg) or vehicle were measured in Sprague Dawley rats. The behaviour of treated animals was measured as moving time (A, B) or distance travelled (C, D) over a period of 120 min. Statistically significance ($p < 0.05$) against the effects of vehicle is shown as *$p < 0.05$ or ****$p < 0.0001$ for the same time-point and was calculated using unpaired t-test. Values are presented as Mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

The locomotion parameter was further subdivided into the parameters moving time and distance gain a more detailed picture of the drug-induced effects (Fig. 49 A-D). Before administration of opioids or vehicle, no behavioural differences were observed between the animal groups (Fig. 49 A-D). After acute administration of UTA1003, significantly reduced moving time compared to the vehicle group at all time-points (unpaired t-test; $t(7) = 2.42$; $p < 0.05$), while moving distance was unaffected (Fig. 49 A, C). On the other hand, morphine significantly reduced both moving time (unpaired t-test; $t(8) = 18.60$; $p < 0.0001$) and distance (unpaired t-test; $t(7) = 2.91$; $p < 0.05$) compared to vehicle, although this effect was restricted to the 30 min-post administration time point. In contrast to UTA1003, morphine increased moving distance (unpaired t-test; $t(6) = 3.17$; $p < 0.05$) 15 min after drug administration (Fig. 49 B, D), while for all other time points no behavioural changes were detected compared vehicle-treated animals (Fig. 49 A-D).

Rearing is a complex behaviour, controlled by the hippocampal formation of the brain (205,207). It is an exploratory, that can be affected by anxiety or escape behaviours and can also increase as a result of opioid withdrawal symptoms (205,206,207,580). Reduced rearing time was observed from 15 to 60 min post-administration of both UTA1003 (e.g. at 15 min; unpaired t-test; $t(10) = 2.24$; $p < 0.05$) or morphine (e.g. at 15 min; unpaired t-test; $t(8) = 3.51$; $p < 0.01$) (Fig. 50 A, B). No rearing time differences were observed between vehicle-, morphine- or UTA1003-treated animals 120 min after drug administration (Fig. 50 A, B). However, both UTA1003 (e.g. at 15 min; unpaired t-test; $t(9) = 3.83$; $p < 0.01$) and morphine (e.g. at 15 min; unpaired t-test; $t(8) = 2.76$; $p < 0.05$) showed significantly reduced rearing numbers until 120 min post-administration (Fig. 50 C, D). Thus, UTA1003 and morphine both reduced overall rearing activities until 2 hours post-administration, while the vehicle-treated animals did not show any significant changes over the observation period (Fig. 50 A-D).

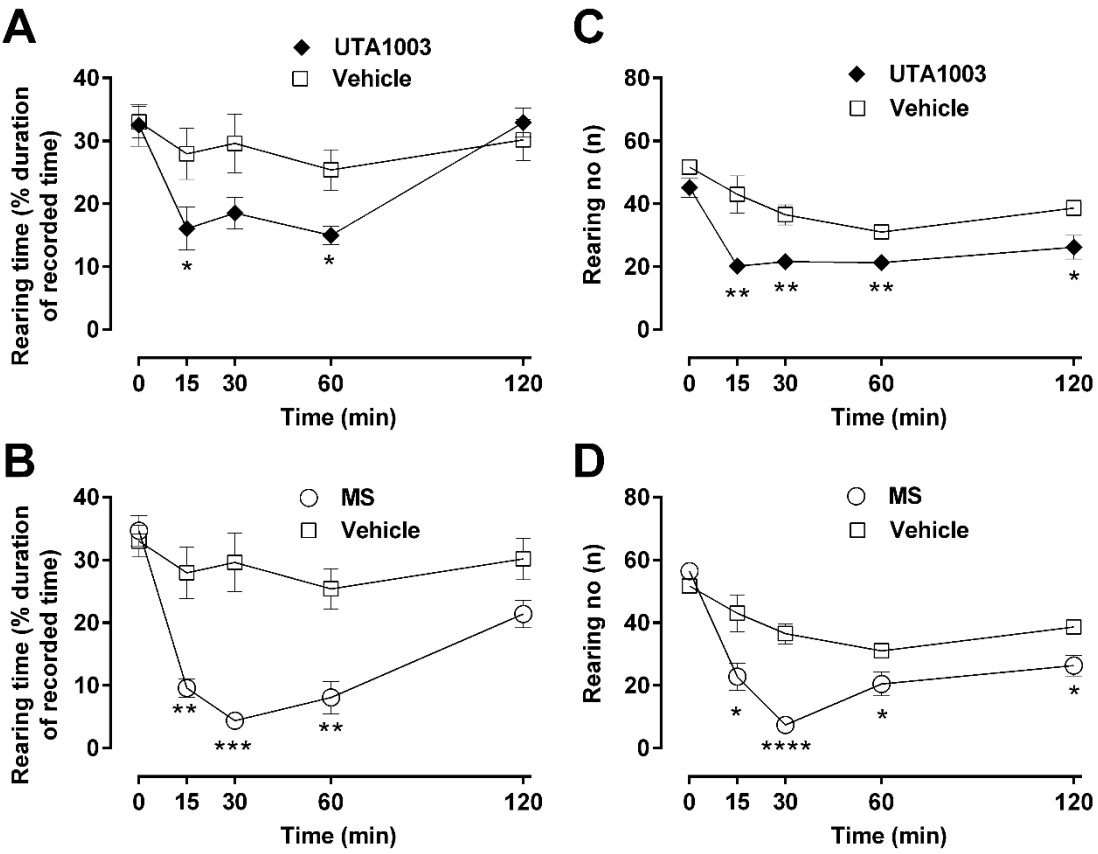


Figure 50. Rearing behaviour after acute UTA1003 and morphine administration. Open-field rearing behaviour after acute subcutaneous injections of UTA1003 (24.6 mg/kg), morphine (3.0 mg/kg) or vehicle was measured in Sprague Dawley rats. Opioid-induced behavioural changes were measured as rearing time (A, B) or rearing numbers (C, D) over a period of 120 min. Statistically significant ($p < 0.05$) differences were compared against the effects of vehicle for the same time-point are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$ and were measured using unpaired t-test. Values are presented as Mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

To investigate these opioid-induced effects on spontaneous behaviour further, rotation or turning behaviour was measured, which is generally believed to an indicator of movement coordination (581). In the present study, no differences were observed between clockwise and counter-clockwise rotations for both vehicle- or morphine-treated animals (data not shown). Similarly, total rotational activities (sum-total of clockwise and counter-clockwise rotation numbers or time) remained unaffected by UTA1003 over the entire observation period (Fig. 51

A, C). In contrast, morphine significantly reduced rotation numbers (unpaired t-test; t (9) = 2.97; $p < 0.05$) and time (unpaired t-test; t (9) = 2.32; $p < 0.05$) 30 min after injection (Fig. 51 B, D) in line with its maximal antinociceptive effect. Beyond this time point, rotation numbers in morphine-treated animals increased back to the basal levels over the 120 min observation period (unpaired t-test; t (7) = 3.00; $p < 0.05$) post-injection (Fig. 51 D).

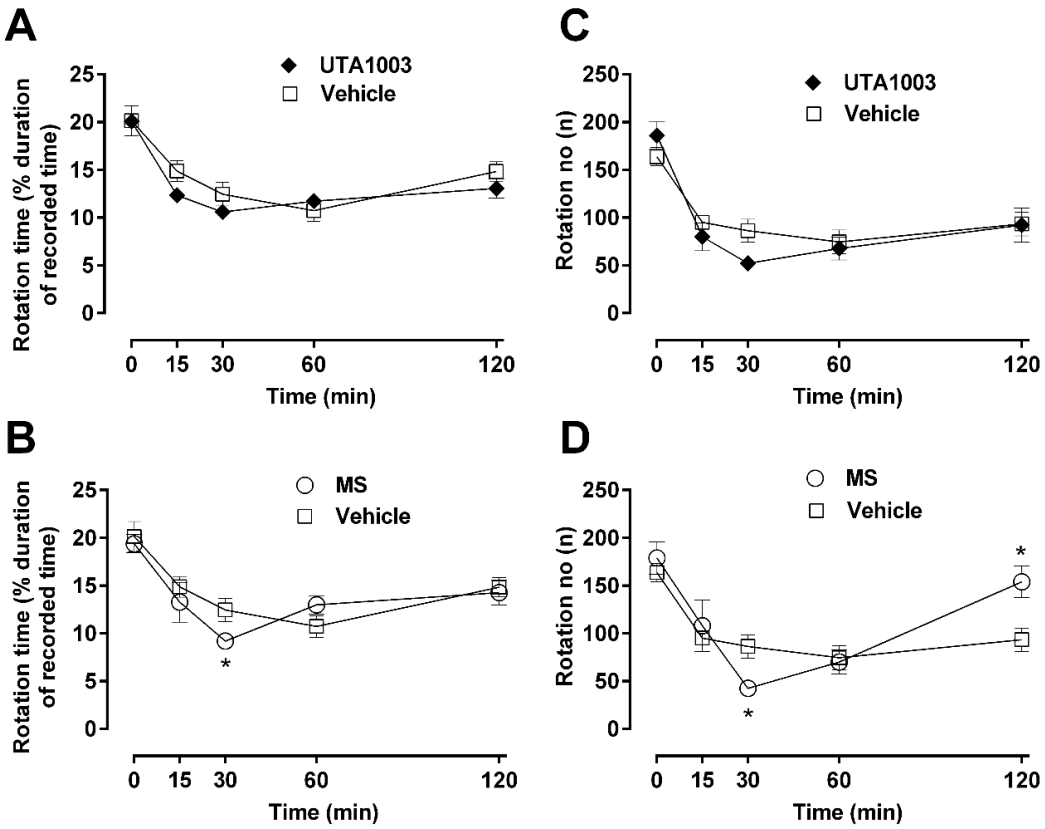


Figure 51. Time-dependent turning behaviour after acute administration of morphine and UTA1003. Effects on turning or rotation after single subcutaneous injections of UTA1003 (24.6 mg/kg), morphine (MS, 3.0 mg/kg) or vehicle were measured in Sprague Dawley rats. The activities of treated animals were measured using an open field test over a period of 120 min. Opioid-induced behavioural changes, such as rotation time (A, B) or rotation numbers (C, D) were compared against the vehicle. Statistically significant ($p < 0.05$) differences at the same time-points compared to vehicle-treated animals are indicated with an asterisk (*) and were calculated using unpaired t-test. Values are presented as Mean ± SEM (n = 6). Error bars are present in all graphs but are sometimes too small to be visible.

The motor behaviour of these animals was further investigated to check out the prevalence of anxiety among these animals (Fig. 52). Place-preferences in the open-field arena is a behavioural marker for anxiety and locomotion (450,481). In the current study, vehicle-, UTA1003- and UTA1003 / morphine-treated animals showed no differences with regards to basal place-preferences over the entire observation period (Fig. 52 A, C, D). On the other hand, morphine-treated rats spent significantly less time between 15 (one-way ANOVA; $F(4, 23) = 7.38$; $p < 0.05$) and 60 min (one-way ANOVA; $F(4, 23) = 7.38$; $p < 0.01$) after administration, which indicates a sign of anxiety among the morphine-treated animals (Fig. 52 B). A reduction of travelled distance at 60 min post time-point (one-way ANOVA; $F(4, 23) = 3.91$; $p < 0.05$) was also observed (Fig. 52 B) suggestive of morphine-induced anxiety.

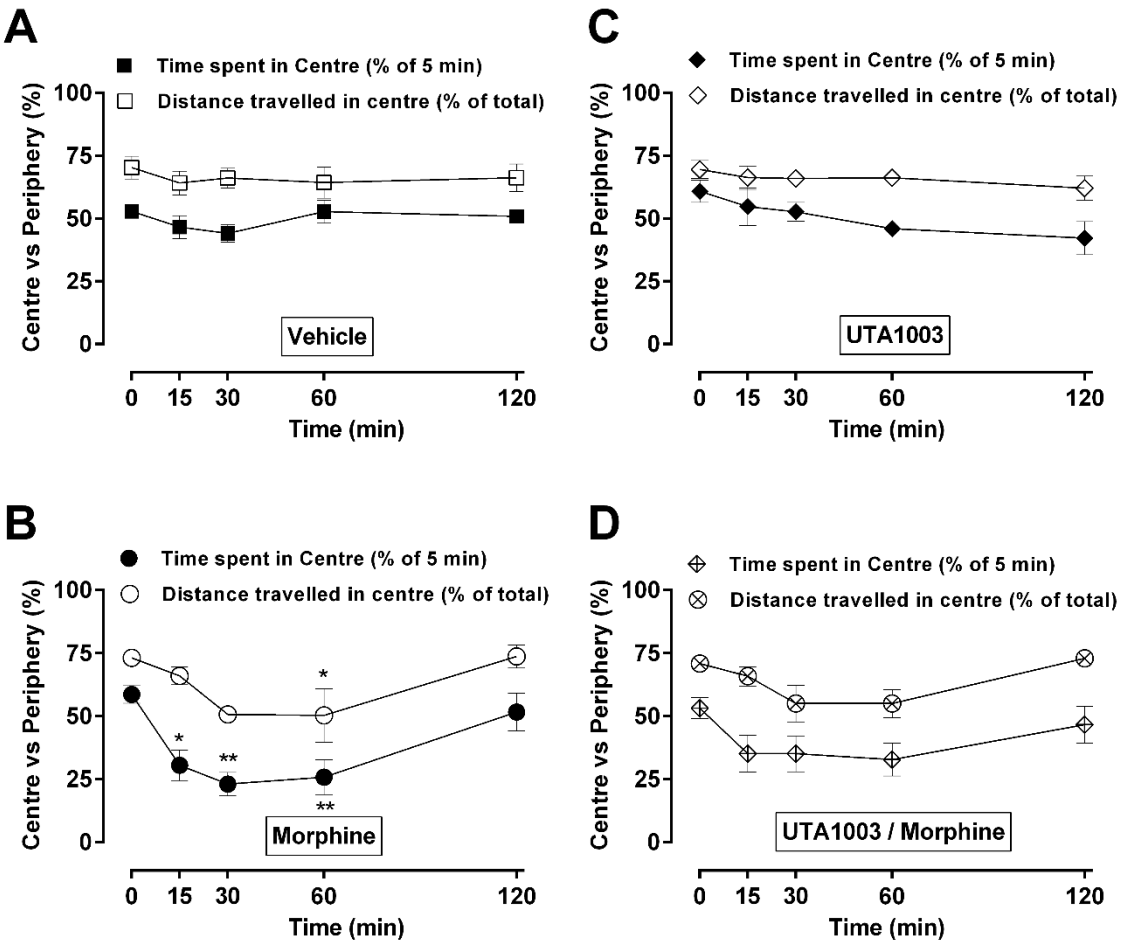


Figure 52. Place-preferences after acute administration of opioids. Effects on time spent in the centre (% of total observation period, 5 min) or distance travelled in the centre (% of total distance travelled by the animal) after single subcutaneous injections of vehicle (A), morphine (MS, 3.0 mg/kg) (B), UTA1003 (24.6 mg/kg) (C) or UTA1003 (24.6 mg/kg) / morphine (3.0 mg/kg) (D) were measured in Sprague Dawley rats. The activities of treated animals were measured using an open field test over a period of 120 min. Statistically significant ($p < 0.05$) differences compared to the basal behaviour of every group of animals are indicated with an asterisk (*) and were calculated using one-way ANOVA with a Sidak's multiple comparison *post hoc* tests. Values are presented as Mean ± SEM (n = 6). Error bars are present in all graphs but are sometimes too small to be visible.

7.3.5. Effect of chronic UTA1003 treatment on motor behaviour

In addition to the acute effects of opioid treatment on behaviour, motor behaviour was also determined during long-term treatment of UTA1003. In this study, animals were treated with morphine, UTA1003 or the combination of UTA1003 / morphine on a twice-daily dosage-regimen over a period of eight consecutive days. Motor behaviour was measured daily as locomotion, rotation and rearing activities at 30 min post-administration using an open-field arena (Fig. 53).

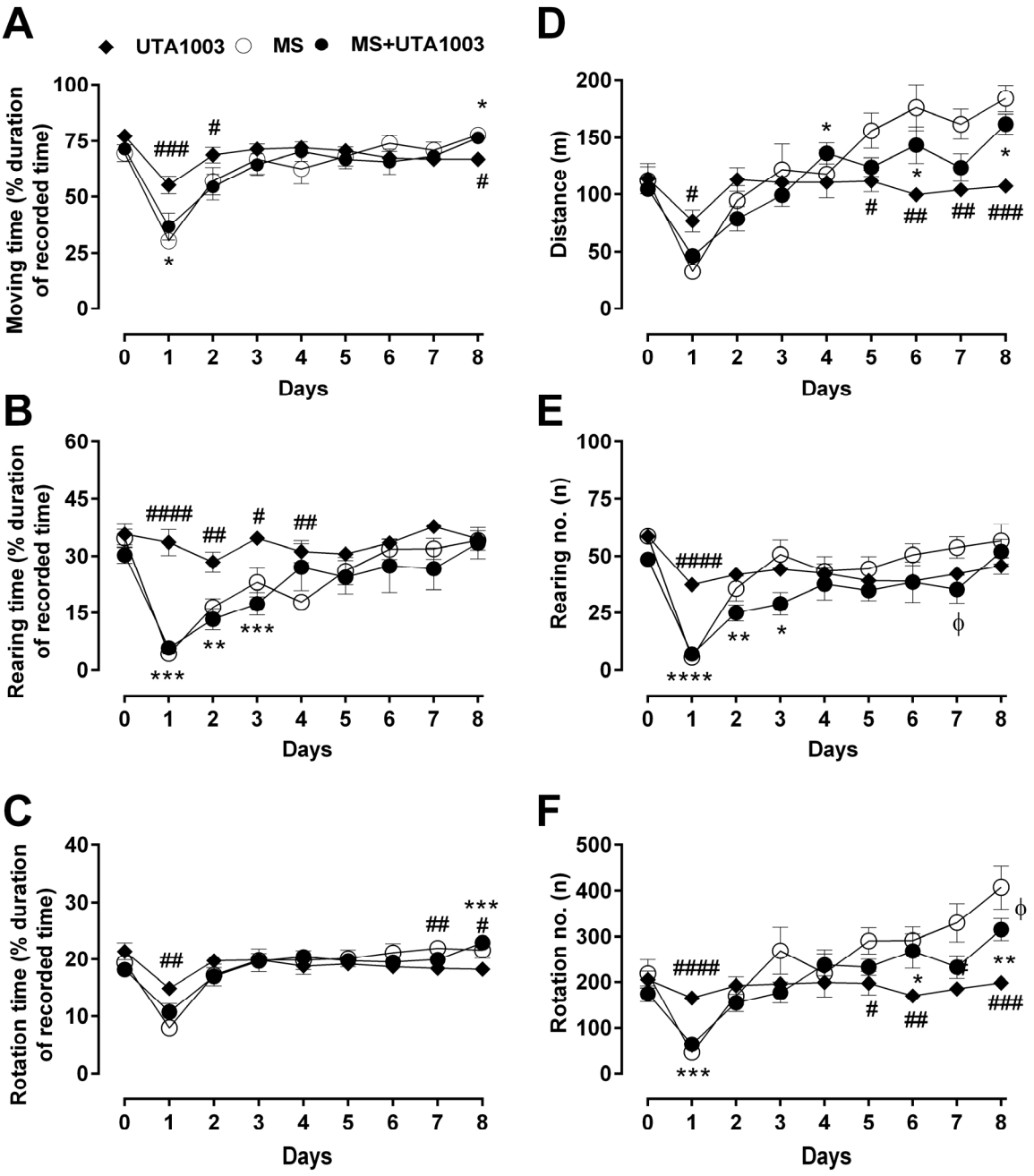


Figure 53. Motor behavioural effects of chronic administration of morphine and UTA1003. Open-field motor behavioural effects after twice daily subcutaneous injections of UTA1003 (24.6 mg/kg), morphine (MS, 3.0 mg/kg) or their combination were measured in Sprague Dawley rats. Opioid-induced behavioural activities were measured daily at 30 min post-injection over a period of 8 days, as described in methods. Several behavioural parameters, such as moving time (A), rearing time (B), rotation time (C), distance travelled (D), rearing numbers (E) or rotation numbers (F) were measured. The blocked diamond, open circle and closed circle symbols show the values of UTA1003, MS and MS+UTA1003 respectively. Statistically significant ($p < 0.05$) differences for the same time-point between the effects of MS and UTA1003 are shown as ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ or $^{\#\#\#\#}p < 0.0001$), between the effects of UTA1003 and MS+UTA1003 are shown as ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$), or between the effects of MS and MS+UTA1003 are shown as ($^{\phi}p < 0.05$) and were calculated using unpaired

t-test. Values are presented as Mean ± SEM (n = 6 animals per group). Error bars are present in all graphs but are sometimes too small to be visible.

To compare the overall motor behavioural activities among different groups of animals (as shown in Fig. 53), the effect of morphine was first compared against UTA1003, where morphine significantly reduced moving time on the first (unpaired t-test; $t(9) = 5.50$; $p < 0.001$) and second days of treatment (Fig. 53 A). Beyond this, higher moving time was observed on day 8 in morphine-treated animals (unpaired t-test; $t(9) = 3.18$; $p < 0.05$) (Fig. 53 A). Moving distance, which is a reliable parameter for exploration or locomotor activities, morphine-induced suppression was observed for this behaviour on day 1 (unpaired t-test; $t(9) = 3.97$; $p < 0.01$) (Fig. 53 D), while higher moving distance was noticed from day 5 (unpaired t-test; $t(7) = 2.56$; $p < 0.05$) to 8 (unpaired t-test; $t(7) = 7.47$; $p < 0.001$) (Fig. 53 D). Thus, morphine-induced biphasic locomotor activities (Fig. 53 A, D), that were previously described (191,193,441). Similar to moving distance, morphine-induced suppression of rotation time on day 1 (unpaired t-test; $t(10) = 4.05$; $p < 0.01$), but hyper-excitation between days 7 and 8 (unpaired t-test; $t(10) = 2.30$; $p < 0.05$) (Fig. 53 C). Likewise, morphine-induced lower rotation numbers than UTA1003 on day 1 (unpaired t-test; $t(6) = 10.57$; $p < 0.0001$), but higher rotation numbers from day 5 (unpaired t-test; $t(8) = 2.41$; $p < 0.05$) to 8 (unpaired t-test; $t(6) = 6.66$; $p < 0.001$) (Fig. 53 F). Morphine also significantly reduced rearing time compared to UTA1003 between days 1 (unpaired t-test; $t(9) = 8.65$; $p < 0.0001$) and 4 (unpaired t-test; $t(8) = 3.89$; $p<0.01$). Beyond that, no differences were observed between the effects of morphine and UTA1003 for the next four days (Fig. 53 B). In contrast to rearing time, morphine-induced lower rearing numbers only on day 1 (unpaired t-test; $t(8) = 10.42$; $p < 0.0001$), implying an overall shorter duration of rearing (i.e. seconds per rearing) between days 2 to 4 (Fig. 53 B, E).

In contrast to morphine, UTA1003 induced almost no changes to basal behaviour (day 0) for all parameters over the course of the 8 day observation period (Fig. 53 A-F).

The behavioural differences between the UTA1003 treated animals and animals treated with UTA1003 / morphine combination were further compared using the open-field test (Fig. 53). The UTA1003 / morphine co-treatment reduced moving time compared to UTA1003 on day 1 (unpaired t-test; $t(10) = 2.58$; $p < 0.05$), but induced increased effects on day 8 (unpaired t-test; $t(10) = 2.80$; $p < 0.05$) (Fig. 53 A). On the other hand, differential moving distance was observed between the groups on days 4 (unpaired t-test; $t(8) = 2.55$; $p < 0.05$), 6 and 8 (unpaired t-test; $t(9) = 5.60$; $p<0.001$), indicating UTA1003 / morphine co-treatment induced hyper-excitation of locomotion on these days (Fig. 53 D). In contrast to locomotor parameters, rearing time and numbers showed similar behavioural pattern over the course of 8 day period. UTA1003 / morphine co-treatment showed reduced rearing time and numbers than UTA1003 treatment from days 1 to 3 (e.g. day 1: unpaired t-test; $t(7) = 6.73$; $p<0.001$ (rearing time); $t(7) = 9.46$; $p < 0.0001$ (rearing numbers)), with no statistical differences observed between days 4 and 8 (Fig. 53 B, E). The rotation time of animals with UTA1003 / morphine treatment showed similar behaviour to UTA1003 treated animals every day except day 8 (unpaired t-test; $t(10) = 5.60$; $p < 0.001$) (Fig. 53 C). In contrast, rotation numbers of the UTA1003 / morphine treatment group were significantly lower than the UTA1003 treated group on day 1 (unpaired t-test; $t(7) = 7.80$; $p < 0.001$) (Fig. 53 F). Noticeably, UTA1003 / morphine induced increased rearing numbers on days 6 (unpaired t-test; $t(8) = 2.98$; $p < 0.05$) and 8 (unpaired t-test; $t(7) = 4.19$; $p < 0.01$) (Fig. 53 F).

To investigate the differences between the morphine-treated animals and animals treated with the combination of morphine / UTA1003, the behavioural activities were further compared and similar activities were observed from day 0 to day 8 (Fig. 53 A-F). Although the drug combination showed an overall trend of reduced distance, rearing and rotation numbers, these effects were mostly statistically non-significant (Fig. 53 D-F). The only significant differences between these groups were observed for rearing numbers on day 7 (unpaired t-test; $t(9) = 2.34$; $p < 0.05$) and rotation numbers on day 8 (unpaired t-test; $t(7) = 3.09$; $p < 0.05$) (Fig. 53 E, F).

7.4. Discussion

Long-term treatment with clinical opioids such as morphine is associated with a loss of therapeutic potential as analgesic tolerance and various types of behavioural side-effects manifest. Currently, all effective clinical analgesics are MOP receptor agonists (22,568,569) and unfortunately, opioid-induced adverse effects are typically transmitted via this receptor (23,24,25). Therefore, novel mixed activity ligands, especially MOP and DOP receptor agonist ligands have come into focus, as these ligands promise to produce less antinociceptive tolerance than conventional, clinically used opioids (150,151,155,160,171,178,184,337,346).

I previously described a novel opioid UTA1003 that is structurally based on the reference compound UFP-505 (349,354). It shares with UFP-505 a MOP receptor agonist / DOP receptor partial agonist *in vitro* specificity profile but displays higher solubility in aqueous solutions compared to UFP-505 (unpublished data, chapter six). The present study investigated the antinociceptive potential of UTA1003 as well as its effects on motor behaviour. I tested UTA1003, morphine and their combination for repeated treatment over a period of eight days to assess their effects on the development of antinociceptive and behavioural tolerance, to highlight functional differences between these compounds.

Subcutaneous administration of morphine in the current study produced maximum antinociception 30 min after drug injection, similar to results described previously (137). UTA1003 showed a mild antinociceptive response, which was significantly ($p < 0.001$) better than UFP-505 at 30 min post injection, although previous reports did not describe any antinociceptive activity of UFP-505 after subcutaneous administration (354). In the present study, repeated morphine treatment induced antinociceptive tolerance from day 2-4, comparable to previous studies (137,171). Importantly, I showed that the combination of

UTA1003 / morphine reduced antinociceptive tolerance of morphine which was comparable to previous studies, that used morphine in combination with a DOP antagonist (122,123,124,125,279). In the present study, the UTA1003 / morphine combination did not prevent morphine tolerance on day 3 (in the tail-flick assay) but maintained nearly 50 % antinociception levels over the remainder of the 8 day period. This 50% antinociceptive effect was statistically significant compared to basal antinociception (day 0). Consequently, drugs like UTA1003 could be developed as a co-treatment with clinically used opioids, irrespective of their intrinsic antinociceptive activities.

It has to be noted that the present study used identical molar equivalent doses of UFP-505 and UTA1003, which were 13 times higher than an equimolar morphine dose. Therefore, UTA1003 appears to be only a weak antinociceptive compound after subcutaneous administration in comparison to morphine. In principle, this could be interpreted a consequence of its peptidic structure, as peptides are known to poorly cross the blood-brain barrier (BBB) (582). However, my data show that UTA1003 suppressed motor behaviour to some extent, similar to morphine. In addition, an approximately five times lower dose of UTA1003 (5 mg/kg/day, once daily) appeared to reduce oxidative damage in the hippocampus of severely depressed rats (unpublished data, Xin Yin), which supports the idea that UTA1003 can penetrate the BBB. This interpretation supports a previous report where several other opioid peptides (e.g. Dmt-DALDA, ADAMB and MZ-2) were described to cross the BBB after systemic administration in animals (583).

In the present study, UTA1003 induced significant levels of antinociception between 30 and 120 min post-administration in the tail-flick test, in contrast to the hot-plate assay, where it showed the highest efficacy 15 to 60 min post-injection. This difference could indicate that the antinociceptive effects of UTA1003 are preferentially supra-spinally mediated, as the tail-flick test measures predominantly spinal-mediated nociception, while the hot-plate assay largely measures a supra-spinal-mediated nociception (135,136,584). The present study deliberately employed two different assays to assess antinociception since the tail-flick assay reportedly overestimates morphine-induced antinociception. Consequently, the use of multiple antinociception tests within the present study aimed to exclude this bias, as previously suggested (136). In addition, a comparably low dose of morphine (3 mg/kg) was used to induce antinociception (138,140,144,145,311), to avoid the tail-flick assay-based overestimation of morphine-induced antinociception (136). The present study showed that the combination of UTA1003 and morphine clearly prevented antinociceptive tolerance when using the hot-plate assay. It is well known that morphine uniformly distributes within the brain stem and spinal cord after systemic administration and therefore its antinociception is seen to be mainly mediated by a spinal response (585,586,587,588). On the other hand, my current data suggest that antinociception of UTA1003 is supra-spinally-mediated since UTA1003 / morphine co-treatment clearly prevented morphine tolerance in the hot-plate assay. Therefore, the combination of morphine and UTA1003 more effectively reduces peripherally mediated than centrally mediated antinociceptive tolerance. This hypothesis could be tested by the tool compound methylnaltrexone that blocks peripherally mediated opioid effects without affecting centrally mediated effects (337,589). As this experiment was beyond the scope of my current study, future studies could use methylnaltrexone pre-treatment to investigate whether UTA1003 induces central or peripheral responses.

Behavioural effects can be measured as changes to motor behaviour, physical dependence or place preference by measuring locomotor activities, movement coordination, exploratory behaviour, naloxone precipitated withdrawal symptoms, reward or drug-seeking behaviour (193,337,346,441,590). The present study assessed motor behaviour simultaneously with antinociception in the same animals as described previously (191,192,193,194). Morphine produced biphasic effects on locomotor activities after both acute and repeated administration in line with previous reports (191,193,441). Repeated treatment with morphine or the combination of morphine / UTA1003 initially produced similar levels of hypo-activity with subsequent recovery (tolerance) and hyperactivity as described previously (unpublished data, chapter three). In contrast, UTA1003 showed only a mild but significant suppression of motor activity on the 1st day that returned to basal levels over the 8 day observation period. Surprisingly, co-treatment of UTA1003 / morphine prevented morphine-induced antinociceptive tolerance, although it did not affect the biphasic morphine-induced changes to locomotor behaviour. Since locomotor activities are controlled by both opioidergic and dopaminergic neurotransmission systems, I speculate that UTA1003 mediated effects are mostly regulated by opioidergic neurons, with negligible effects on dopaminergic neurons, although I did not conduct any specific experiment to validate this hypothesis.

Although rearing activity is thought to be a reliable parameter to assess exploratory behaviour (192,205,210), the reduced rearing may be caused by a multitude of effects that include lack of interest, motor impairment, depression or anxiety (206). In this study, repeated morphine-exposure suppressed rearing indicates reduced animal exploratory behaviour. This effect was reported previously (462) and was interpreted to reflect morphine-induced sedation or drowsiness in the clinic (451,454). The current study observed a significant morphine-induced reduction of rearing behaviour indicative of reduced exploratory behaviour. However, repeated

morphine treatment over only a few days was reported to induce apoptosis in cortex and amygdala (591). Since the motor behaviour is known to be controlled mainly by the cortex, amygdala and hippocampus (592,593,594,595), this effect could theoretically be responsible for the observed motor impairment in these animals. However, this data overall do not support this hypothesis, as the animals showed hyper-excitation, especially at later time points which cannot be reconciled with an apoptotic loss of neurons that should be more prevalent after longer treatment intervals. Morphine-induced reduced rearing could also be a result of drug-induced anxiety-like or depressive-like behaviour. However, this possibility stands in stark contrast to previous studies that reported both anxiolytic (596,597) and anti-depressant effects of morphine (598,599,600). The association between morphine and its anti-depressant effect was not observed in my study, which instead indicates morphine-induced anxiety may have been present in our animals as shown in previous studies (601,602). Therefore, based on my results, it cannot finally be determined if the lack of rearing activities of morphine-treated rats in my experimental system is a result of reduced exploratory behaviour or anxiety.

As previously reported, morphine also reduced turning behaviour (459,460), which is a complex behaviour that is mainly mediated by the dopaminergic system (199,200,457). Abnormal turning or rotational behaviour can occur due to an imbalance of nigrostriatal function that controls dopaminergic neurotransmission (581). These dopaminergic neurons are connected to GABAergic neurons in the nuclear accumbens and substantia nigra, which therefore implicates these neurons in the control of turning behaviour (603,604). The observed suppression of turning behaviour after acute treatment of morphine replicates two earlier studies that reported similar levels of suppression (459,460). I show that morphine-induced rotation numbers are also subjected to tolerance after repeated administration (Fig. 53), which follow a similar pattern to morphine-induced antinociceptive tolerance (Fig. 48).

Although the described effects of morphine and UTA1003 on behaviour appear significant and replicate previous studies, my results raise significant concerns. This study observed significant reductions of moving distance, rearing and rotation numbers 30-120 min after exposure to vehicle (10% DMSO, 90% saline). These surprising results support a previous study that reported similar effects after intracerebroventricular (i.c.v.) and oral administration of DMSO in mice (605). Since DMSO metabolites can reduce motor behaviour (606), I have to assume that DMSO in my study may have affected the motor behaviour of my vehicle-treated animals, although so far information related to subcutaneous treatment was not included in the previous study (605).

The vehicle-induced reduction of motor activities in the present study may also be an indication of anxiety-related behaviour of these animals as repeated open-field measurement may induce behavioural changes in a time-dependent manner (607). After the first exposure in an open-field arena, animals may have an increased level of anxiety with increased locomotion and rearing behaviours, which normally disappear after subsequent testing (607). Although the animals used in this study were habituated in the testing room for 2 h before the start of the daily experiments and an additional five minutes pre-exposure to the open-field apparatus before basal motor behaviour was recorded, the possibility of anxiety-related behavioural changes cannot be excluded. Since all animals were treated under the same experimental conditions, statistical comparisons at the same time-point between the vehicle and opioid-treated animals should exclude any possible environmental bias. Decreased locomotion in the central area of the open-field indicates an anxiety-like behaviour (450,608,609,610,611). However, the vehicle-treated animals of this study did not show any changes in locomotion in the central area of the open-field over the whole observation period (Fig. 52 A). Therefore, vehicle mediated effects are unlikely a sign of environment-or DMSO-induced anxiety

(605,612,613) and this effect has to be investigated in much more detail, using specialised behaviour paradigms to understand its origin. At present, however, this effect has to be seen as a major confounding factor of the current study.

In summary, my results illustrate that UTA1003 produced no antinociceptive tolerance and prevented to some extent the morphine-induced antinociceptive tolerance after repeated administration. I also show that UTA1003 induced less motor suppression than morphine, although it did not interfere with morphine-induced suppression or hyper-excitation of motor behaviour after co-treatment of UTA1003 / morphine. Therefore, subsequent studies will have to focus on a detailed pharmacokinetic study of UTA1003 and its structural analogues to increase its efficacy with the systemic administration in preclinical models. Overall, UTA1003 appears to represent a promising lead compound with an advantageous toxicity profile for future studies for the development of potent analgesics with reduced antinociceptive tolerance and other adverse effects.

CHAPTER EIGHT

Summary

8. Summary

Effective measures to ensure efficacy and safety of long-term use of opioids regarding pain-relief are not well established in the clinic (249,614). In addition, abuse of opioids (both prescription and non-prescription opioids) has increased significantly in many countries, which is of great concern to regulatory agencies worldwide (615,616,617,618,619). This study intended to develop strategies to reduce opioid-induced antinociceptive tolerance and motor behavioural adverse effects over a period of long-term opioid treatment. After a general introduction to the topic (chapter one), the second and third chapters described an effective dosing strategy to reduce morphine-induced adverse effects. Chapter four described age-dependent variations of morphine-induced antinociceptive and behavioural effects, which are important for dose-calculations of morphine for use in the elderly population. To highlight another opioid-induced adverse event, chapter five evaluated the role of opioid receptors on insulin homeostasis. Finally, chapters six and seven described the pharmacological profiles of novel opioids with mixed activity on multiple receptors generated at the University of Tasmania as well as their effects on the two main adverse events associated with opioids: antinociceptive tolerance and altered motor behaviour.

The present study together with previous reports show that an optimised opioid dosage-regimen should consider: (i) the patient need (severity of pain, disease condition) (413,483,620,621), (ii) dose, (iii) frequency of daily dosing, (iv) duration of treatment, (v) opioid formulation (e.g. morphine- base, sulphate or hydrochloride), (vi) dosage form (immediate/sustained release formulation), (vii) route of administration (137,622), (viii) patient age (e.g. G protein-coupled kinases and β-arrestin expressions decrease with advanced age) (507), (ix) genetic factors (poor/rapid metaboliser of opioids) (424,425,426,427,428), (x) polypharmacy (multiple drug intake), (xi) multi-morbidity (such as patients with diabetes mellitus, obesity), (xii) drug-drug

interactions (423,424) and (xiii) the behaviour of individual patients (addiction or dependency on non-prescription narcotics or alcohol) (623,624,625,626).

Surprisingly the results of the present study indicate that the 'start low and go slow' dosage regimen of opioids, proposed by all current clinical guidelines, does not appear to provide adequate pain-relief and furthermore facilitates tolerance induction. Although this study did not determine how much higher a morphine dose should be to provide effective antinociception without inducing adverse effects, my results indicate that the duration of treatment is an important parameter that further affects dosage-estimations. My results are supported by clinical and preclinical studies, which agree that long-term use of low-doses of opioids increases pain-sensitivity (hyperalgesia). This would suggest that higher opioid doses are required for effective analgesia and to avoid hyperalgesia (180,577,578,579).

My results suggest that to avoid some of the opioid-induced adverse effects, optimised opioid dosing should include one or several of the following strategies: (i) a high dose at the start of treatment, (ii) reduced frequency of daily dosing, (iii) larger dose-increments after tolerance manifestation, (iii) co-treatment of an opioid (e.g. morphine) and a DOP receptor antagonist (e.g. naltrindole), (iv) combination of a clinical and an opioid with mixed selectivity on different opioid receptors (e.g. UTA1003), (v) rotation of opioids, (vi) changing administration routes, (vii) reduction of polypharmacy if possible, (viii) reduction of the use of psychoactive agents (e.g. benzodiazepines and alcohol) and (ix) age-related dose-adjustments (Fig. 54).

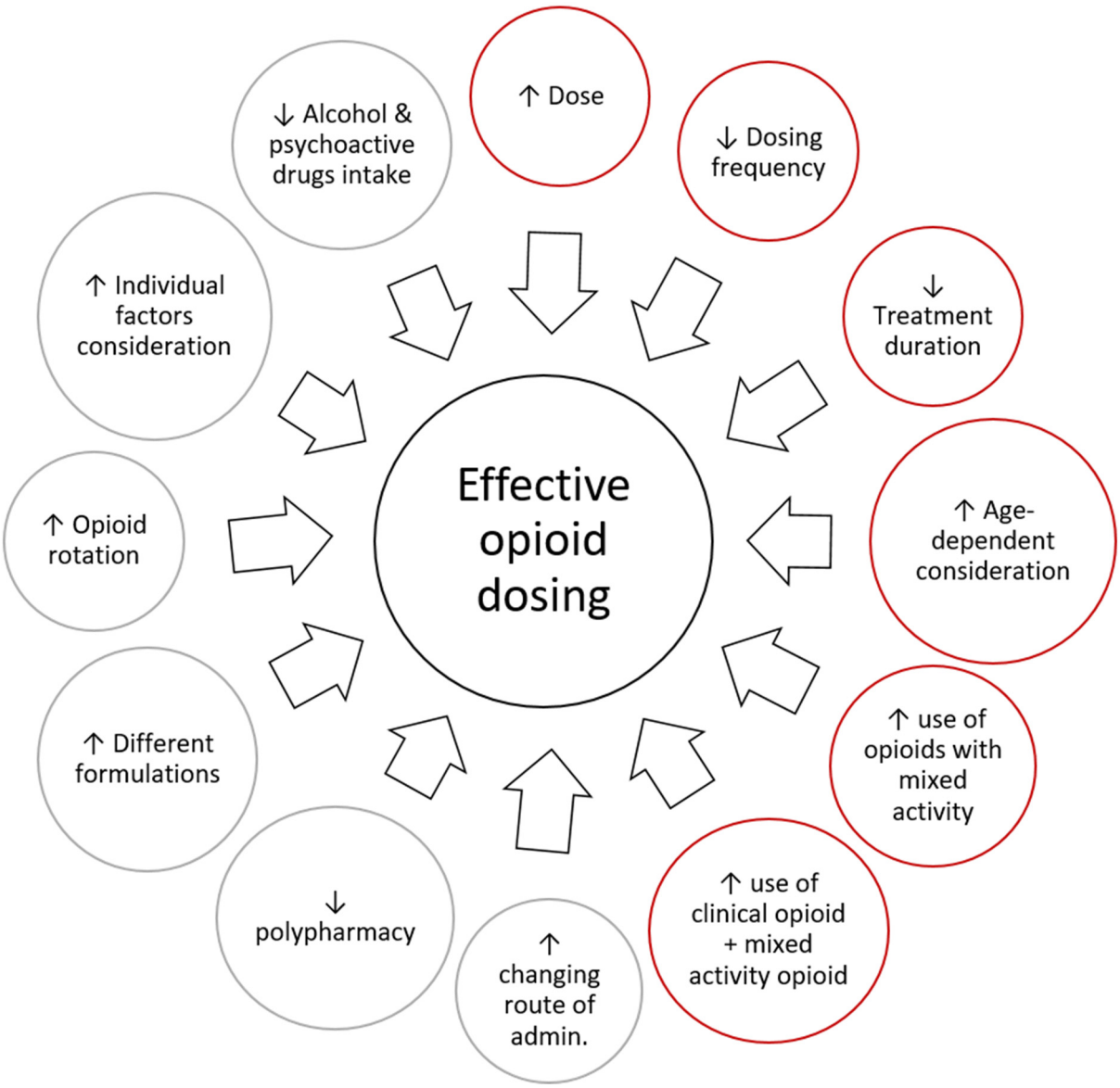


Figure 54. Strategies for optimised opioid dosing to reduce adverse effects. The information obtained from the current study (red circles) was combined with the reported literature (grey circles).

Motor behavioural effects are common adverse effects of long-term opioid treatment. Activation of the MOP receptor by an agonist (e.g. morphine) increases dopamine levels in the brain (627,628), which are likely to decrease opioid-induced locomotor, rearing and turning behaviours (629) (Fig. 55). This study demonstrated that motor behavioural suppression by a MOP receptor agonist is paralleled by antinociceptive tolerance levels. Importantly, the hyper-

excitatory motor behaviour of morphine appears to be directly linked to its antinociceptive tolerance. Therefore, the motor behavioural measurement could potentially alleviate the need for painful nociception tests (e.g. tail-flick, hot-plate tests), although further verification of this hypothesis using additional opioid ligands is required.

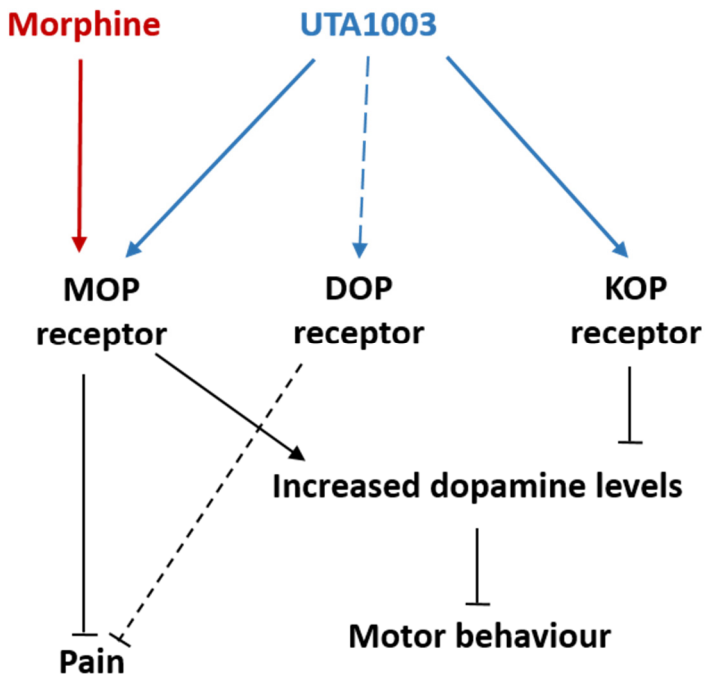


Figure 55. Effects of UTA1003 and morphine on antinociception and motor behaviour. The receptor selectivity profile of morphine has been adopted from IUPHAR-BPS Guide to Pharmacology (12,630,631,632). In this study, UTA1003 or morphine were not assessed for their pharmacological efficacy on dopamine levels in the brain. The role of DOP receptor on motor behaviour is obscure (633,634,635). This figure represents the results of the present study combined with the reported literature. Keys: ↓: stimulate; ⊥: inhibit or antagonise.

Although elderly individuals are significantly more likely to use prescription opioids compared to younger individuals, an optimum dose-determination of opioids for this age-cohort is dependent on a multitude of parameters. However, general clinical practice only assesses a limited number of these and use them for necessary dose adjustments. These parameters are mainly related to altered age-dependent ADME characteristics and are further complicated by the prevalent polypharmacy as a consequence of age-related multi-morbidity (636,637,638). In particular, impaired renal function, altered cytochrome P450 activities, reduced GPCR kinase and β-arrestin expression is associated with progressive age, all of which could endanger the

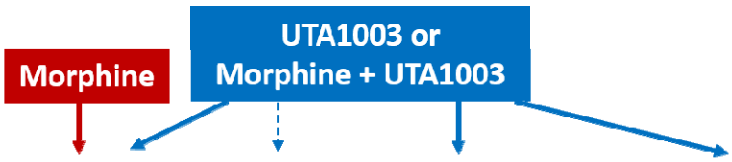
elderly patient to opioid overdosing and subsequent toxicity (425,427,428,507). The present study described a prolonged duration of action of morphine in older, compared to younger animals, which was related to opioid-induced antinociception as well as behaviour. Consistent with altered ADME characteristics, this study also demonstrated that these age-dependent effects were likely based on different residual opioid levels in the brains of older animals. To translate the observations of the present study into the clinic, appropriate opioid dose adjustments for older people should anticipate lower opioid doses, which is in agreement with previous clinical studies (485,486).

Appropriate opioid dosing also depends on the physiological condition of patients that suffer from chronic and metabolic diseases. Type I diabetes (a metabolic disorder) is associated with the degeneration of pancreatic β-cells, which results in decreased plasma insulin levels (516). Type II diabetes is caused by reduced cellular insulin sensitivity and elevated plasma glucose levels (355,356). Both types I and II diabetes are associated with diabetic neuropathy, where patients experience neuropathic pain and therefore require analgesic medications (639). Optimum insulin secretion from pancreatic β-cells and glucose utilisation are required to manage type I diabetes mellitus, which may contribute to the reduced diabetic neuropathy of this patient cohort (640,641,642). The present study showed that MOP or DOP receptor activation induces insulin secretion in a pancreatic cell line, whereas the KOP receptor activation left insulin secretion unaffected (643). From a clinical perspective, MOP or DOP receptor-selective opioids may, therefore, provide some benefits to increase plasma insulin levels but at the same time also potentially interfere with diabetic medication schemes. Although further studies are required to gain more detailed insights into opioid-dependent insulin secretion, this effect should be carefully considered in diabetic patients to provide optimised treatment paradigms and to prevent unwanted adverse effects due to drug-drug

interactions.

Opioids with mixed selectivity profile on multiple receptors produce less adverse effects (tolerance and motor behavioural effects) than morphine or other opioids that are selective for the MOP, DOP or KOP receptors (26,147,443,644,645,646) (Fig. 56). In this study, the novel opioid UTA1003 with a mixed activity profile on different opioid receptors was identified and characterised. UTA1003 appeared as a mild antinociceptive agent after acute subcutaneous administration but induced no antinociceptive tolerance after repeated treatment. More importantly, a combined treatment of UTA1003 and morphine reduced morphine-induced antinociceptive tolerance (Fig. 56). This study indicates for the first time that co-treatment of a clinically used opioid with an opioid with mixed selectivity profile on multiple opioid receptors can reduce the adverse effects of clinical opioids, although further investigations are required to strengthen this concept with regards to other adverse effects as well as for other opioids with mixed selectivity profile on multiple opioid receptors.

In this study, acute UTA1003 treatment showed reduced but not absent behavioural suppression compared to morphine. Interestingly, no behavioural adverse effects were observed after repeated administration of UTA1003 (Fig. 56), which is likely due to its MOP receptor agonist / KOP receptor agonist profile, since KOP receptor activation antagonises MOP receptor-induced behavioural effects (Fig. 55). In contrast to the beneficial effects of co-administration of UTA1003 with morphine on tolerance induction, repeated co-treatment of both drugs could not protect against morphine-induced behavioural effects. As the behavioural effects are complex and controlled by both opioidergic and non-opioidergic (e.g. dopaminergic) neurons (Fig. 55), future studies need to investigate the detailed mechanism why UTA1003 shows differential effects on antinociception and motor behaviour.



Parameters	MOPr agonist	DOPr agonist	Mixed activity on MOPr and DOPr	KOPr agonist
Analgesic tolerance	↑	↑	↓	↑
Motor behaviour	↓↑	?	≈	↓
Constipation	↑	?	↓	≈
Respiratory depression	↑	≈	↓	≈
Physical dependence	↑	≈	↓	≈
Addiction	↑	≈	↓	≈
Itching	↑	≈	≈	≈
Hyperalgesia	↑	↓	≈	≈
Insulin release	↑	↑	?	≈
Euphoria	↑	≈	≈	≈
Dysphoria	≈	≈	≈	↑
Depression	?	↓	≈	↑
Sedation	↑	≈	≈	↑

Figure 56. Expected benefits of the co-treatment of UTA1003 and morphine. Keys: MOPr, DOPr, KOPr and NOPr: μ, δ, κ and non-opioid receptors respectively; "?": unknown/inconclusive evidence; "↑": increased effects; "↓": decreased effects; "≈": no effects. The information presented in this figure represent the results of the present study combined with the published literature in this field (25,140,147,153,172,231,233,645,647,648,649,650,651,652).

In the present study, Sprague Dawley rats were used to assess the antinociceptive and behavioural effects, which could be tested on mice or another animal species. No vehicle-treated animals were used as control groups in chapters 2-4, which is a significant limitation of my study. Future studies should investigate opioid-induced effects by comparing opioid-treated with vehicle-treated (0.9% w/v sodium chloride solution in water) control animals. Thermal nociception assays (tail-flick and hot-plate tests) were only used in this study to assess the

efficacy of the opioids, but the unchanged baseline antinociception and behaviour over two weeks of repeated treatment (chapters 2 and 3) can potentially indicate that the absence of adverse effects like hyperalgesia or allodynia. The selectivity profile of the novel UTA-opioids could be verified by using radio-ligand binding assay or GTPγ^{35}S assay, although the use of cAMP assay potentially helped me to identify the potential ligand for preclinical assessment. A detailed pharmacokinetic study should be conducted in future to understand the effects of UTA1003 and UFP-505, which could not be fitted with the present study design with simultaneous measurements of antinociception and behaviour of the same animals. Further chemical modification in the structure of UTA1003 is warranted in future to increase its efficacy after subcutaneous administration.

Adequate opioid use should maintain long-term antinociception and safety of patients in the clinic and the present study has provided new knowledge on the role of opioid dosing for the induction of antinociceptive tolerance. My study also indicated that detailed motor behavioural testing could have the potential to replace the widely used painful preclinical antinociception testing to investigate antinociceptive tolerance. This potential paradigm shift could significantly reduce of animal suffering in future studies of pain-research. I observed that older animals need less morphine to maintain effective antinociception and behavioural effects, which indicates additional dosage adjustment to avoid toxicity and to ensure safety for the elderly population. I also added additional knowledge to the literature on the role of MOP and DOP receptors on insulin homeostasis, which could be particularly important for opioid dosing adjustments in patients with diabetes. In this study, I identified and characterised a novel ligand UTA1003 with mixed activity profile on different opioid receptors. UTA1003 produced less adverse effects than morphine either alone or after co-treatment with morphine in rats. Therefore, an optimised opioid dosing can also include a combination of an opioid with an

opioid with mixed selectivity profile on multiple opioid receptors to maintain long-term safety for the patients. Finally, this study confirms that targeting multiple receptors is a promising pharmacological strategy to reduce opioid-induced adverse effects.

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Appendix A. Additional behavioural effects of morphine

A.1. Qualitative behavioural effects of morphine

Drowsiness, lethargy, hyperactivity, itching and respiratory depression are common adverse effects of morphine (230,250,251,653). Morphine is also responsible for itching sensation by signalling through heteromers of opioid- and itch-mediating G-protein receptors (253). I observed that some animals showed signs of itching and wheezing over time (Table A1). Consistent with the situation in patients where these effects are not dose-dependent (251), I also could not demonstrate a dose dependency in the rats of the current study.

The detailed information regarding animals, dosage and treatment protocol is described in the *Methods* section of chapter 3 (section 3.2).

Group A Morphine: 5 + 5 mg/kg/day → 10 mg/kg/day

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Home-case activity: Sluggish (-) / Active (+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Itching: absent (-) / present (+)	-	-	-	++	-	-	-	-	-	++	+	++	++	++
Wheezing: absent (-) / present (+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Group B Morphine: 10 + 10 mg/kg/day → 20 mg/kg/day

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Home-case activity: Sluggish (-) / Active (+)	-	-	-	-	-	+	+	+	+	+	-	-	+	+
Itching: Absent (-) / present(+)	-	-	-	-	-	-	-	+	-	-	+	+	+++	+++
Wheezing: Absent (-) / present (+)	-	-	-	-	-	-	-	-	-	-	+	+	+	+

Table A1. Qualitative behavioural effects of daily morphine-treated rats. Homecase-activity, itching and wheezing effects of daily morphine-treated Sprague Dawley rats (n=6 per group) over the course of 14 days (groups A and B) and the effects were measured daily using cautious subjective measurements on the animals in their home-case over a period of 60 min after morphine administration.

Appendix B. Animal ethics approval permit

University of Tasmania
Animal Ethics Committee

ETHICS APPROVAL PERMIT

University of Tasmania
Office of Research Services
Ph: 03 62267283
Fax: 03 62267148
animal.ethics@utas.edu.au

To:	Dr Nick Dietis
From:	Marilyn Pugsley Executive Officer Animal Ethics
Date:	2 April 2014
Project:	**A13864 - The effects of novel bi-functional opioids in analgesia**
Approved on:	2 April 2014
Approval expires:	2 April 2017
1st Annual Report due before:	2 April 2015

Please read this permit carefully as **approval may be withdrawn** for projects that do not comply with the conditions

The Animal Ethics Committee has approved the above project and a copy of the document is attached. The approval is subject to the review and approval of an annual report which is due before the approval anniversary. **Please note this date in your diary.**

This approval constitutes ethical clearance by the Animal Ethics Committee. If this project involves the conduct of a Veterinary Service or Other Animal Service as defined in the Veterinary Surgeons Act 1987 (Tas) and Veterinary Surgeons Regulations 2012 (Tas), it is your responsibility to ensure that the project is conducted in accordance with the provisions of the Act and Regulations. Please contact the Animal Welfare Officer, Dr Sue Ottomanski (6226 7491 or sue.ottomanski@utas.edu.au) to discuss veterinary procedure competency accreditation.

As the Responsible Investigator, you MUST ensure that:

(a) all aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

(b) a full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.

(c) you contact the Animal Welfare Officer, Dr Sue Ottomanski (sue.ottomanski@utas.edu.au) to advise her when and where your experiments will be conducted. Sufficient notice needs to be given so that if the AWO wishes to make an inspection, this can be easily arranged.

(d) That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.

The Animal Ethics Committee is to be promptly notified of any unexpected events which occur during the period of the approved project and impact on the welfare of the animals.

Autopsy should be performed by a qualified veterinarian when animals die unexpectedly. Any foreseeable departure from this requirement must have been outlined and approved in the initial application.

If the project is to continue past the expiry date, a new initial application will need to be submitted. A project can only be approved for a maximum of 3 years.

If the investigation necessitates a Parks & Wildlife permit you are required to send a copy of this permit to the AEC Secretary before commencing work.

Marilyn Pugsley
Executive Officer Animal Ethics

University of Tasmania **Animal Ethics Committee**	
Ethics Number	A13864
Project Name	The effects of novel bi-functional opioids in analgesia
Chief Investigator	Dr Nick Dietis
School	Pharmacy
Person responsible for day-to-day care	
Ethics start date	2 April 2014
Ethics approved to	2 April 2017 (with annual renewal)
Emergency Contact	

Research Services
University of Tasmania
Private Bag 01
Hobart, Tasmania 7001
Tel: (03) 6226 7283
Fax: (03) 6226 7148

animal.ethics@utas.edu.au

University of Tasmania
Animal Ethics Committee

INITIAL APPLICATION FORM

Please submit the completed and **SIGNED** form electronically (hard copies are not required). The AEC process can take up to 8 weeks so please consider this when planning your project start date.

Note:
- The Responsible Investigator and Nominee must be UTAS employees
- This form should be completed using *the Instructions for applying for ethics approval* sheet on the UTAS Animal Ethics website.
- Some questions have associated endnotes which provide essential information and expansions on the questions. It is essential that you read them when completing this form. Hover the curser over references numbers where available in order to see the associated relevant endnotes.

Details of the Project *(Please type in the boxes provided)*

Project Title [1]	The effects of novel bifunctional opioids in analgesia
Responsible Investigator [2]	Dr Nikolaos Dietis, Lecturer in Pharmacological Science, Division of Pharmacy, School of Medicine
School or Discipline	School of Pharmacy
Nominee[3]	Assoc Prof Nuri Guven, Senior Research Fellow, Division of Pharmacy, School of Medicine
Associate Investigators *(include position)*	Alok Paul (PhD student)
Project Commencement Date	14/04/2014
Is this an Honours project or does it incorporate an Honours project?	No

Declaration by Head of School *(in some cases the head of discipline may be more appropriate)*

NB: If the Head of School or Discipline is one of the investigators, an appropriate person must sign the following statement of scientific/educational merit. This will normally be the Head of School or Discipline in a related area.

- I consider that the research investigation or teaching activity detailed in this application is justified on scientific and/or educational grounds;
- I confirm that facilities are available to ensure the welfare of the animals whilst they are in my department;
- I am satisfied that all personnel who will be involved with the proposed investigation are familiar and will comply with all relevant Commonwealth and State or Territory legislation and the requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th edition 2013;
- I shall ensure, as far as I am able, that I will comply with the principles of the Code throughout the investigation.

Head of School or Discipline

Name of Head of School or Discipline: Dr Luke Bereznicki (Deputy HoS)

Signature: ··

Date: 25/02/2014

1. Primary Purpose: Higher Degree Research

- **Teaching** covers all teaching activities and vocational training conducted by and/or involving UTAS staff and/or students including high school and college teaching activities and demonstrations. If you have selected this option, **you must also complete** Attachment A relevant to teaching activities at the end of this document.
- If you have selected **Teaching and Research you must also complete** Attachments A and B relevant to teaching activities at the end of this document.

2. Overview of the Project

It is important to give a clear description in ***plain English*** because not all members of the committee are scientists (maximum ½ page per answer please)

2.1 What is the background that has led to this project?

Pain is a significant burden for many patients with cancer or other chronic pain conditions. Although strong pain-killers like opioids (i.e. morphine) are the mainstay of therapy in most cancer patients, there are frequent side effects. Most importantly, chronic administration of morphine produces a quick onset of 'tolerance', which is displayed as a reduction in pain relief after repeated administration.
A need for new opioid drugs that do not produce tolerance has been the focus of many research groups worldwide, including our own **(1)**, during the last few years. Our work to date has addressed this problem and very recently presented a novel and innovative approach of new drug design **(2)**, which is believed to be an important step forward towards an effective treatment of cancer and chronic pain. We have developed a prototype drug of this type, called UFP-505, and we successfully conducted the first preliminary animal experiments in rodents **(3, 4)**, that showed that UFP-505 reaches the aimed targets of strong pain relief without tolerance after 5 days of repeated treatment.
These promising results comprise the first ever animal data for such a type of drug candidate, that doesn't producing tolerance, and warrants further intense research into the properties of this class of drugs. In collaboration with the School of Chemistry at UTAS we produced newer versions of this novel class of drugs (called UTAS drugs) in order to explore potential improvements.
In this study, we will study the analgesic effects of these novel UTAS drugs in rats in order to obtain the knowledge currently missing from the drugs developed.

2.2 What are the major aims of the project?

In this project, we aim to explore the analgesic effects of the UTAS drugs. More specifically we aim to reach the following objectives:

1. To determine the effect of weight in the analgesia of rats treated with opioids.
2. To determine the acute pain-relief of UTAS drugs in comparison with morphine.
3. To determine the tolerance levels of UTAS drugs in a chronic study.
4. To determine the analgesia of UTAS drugs in different routes of administration
5. To determine the analgesia of UTAS drugs using an orofacial test.
6. To determine the analgesia of UTAS drugs when in simultaneous treatment with drugs used currently for humans (morphine, codeine, oxycodone and fentanyl).
7. To determine how the body copes with the UTAS drugs and how the drugs affect the body after a short-term treatment (pharmacokinetics).

2.3 What are the anticipated benefits that justify this project?

1) This project will provide us with the necessary data for the effectiveness of our novel drug candidates in terms of pain relief, in order to distinguish to the best candidate possible.
2) This project has the potential to generate valuable intellectual property for UTAS through the drug discovery process and also provides a solid platform for collaboration between three Divisions within UTAS (Pharmacy, Chemistry, Psychology) aligned with the UTAS Strategic Research Plan.
3) The results from this project will shed light on the current research area of opioids, contributing to our knowledge on the effects of opioids on cancer pain.

2.4 What will actually happen to the animals?
(This is to provide a context for your project. A more detailed answer is required in Question 5)

The animals will be tested for their nociception levels (e.g. level of stimuli that produces discomfort) with and without treatment with drugs. The testing of nociception will involve placing the animals on two different equipment that assess nociception prior and after their treatment with drugs. In acute

experiments, exposure to drugs will take place only once and animals will be tested during a day. In chronic experiments, exposure to drugs will take place during 8 days and animals will be tested during these days. In tests that involve orofacial testing, animals will be fasted for 12 hours and then be placed in the orofacial test equipment, with or without drug exposure.
For more detailed information, please refer to the procedures in Section 5.

2.5 Which of the following aspects of the code does your project address?

(Please choose an option): Maintaining or improving human and/or animal health and welfare

3. Are precise numbers of animals known? No

In answering this question, please consider potential mortalities associated with transportation, housing, specific transgenic lines etc., that could occur in addition to mortalities as a direct result of your research. (Important note: If additional numbers are anticipated during the course of the research, the AEC must be notified in advance).

- If yes, insert the precise numbers on the table marked 3.1.
- If no, please provide your estimated numbers in Table 3.2.
  (Please note that these numbers can be modified annually)

3.1 Precise Numbers

Please Note: any discrepancies between the number of animals required and the number of animals delivered by a supplier MUST be addressed. If numbers cannot be precise, it may be more appropriate to complete Table 3.2 Estimated Numbers. Full details of the fate of all animals that have been oversupplied must be included in your annual report.

Species *(common and scientific names)*	Strain (if applicable)	Year	No to be used	No to be killed	Source[4]	Location[5]
Rat (Rattus norvegicus)	Male Sprague-Dawley	1	**152**	**152**	**Cambridge**	1 - Tasmania
		2				Choose an item.
		3				Choose an item.
		Total				

Note: In the case of lab animals, in the space below, please advise whether strains are GMO'S, Knockout, Knockin or Mutations.

None of the above

3.2 Estimated Numbers

Note:

Estimating exact numbers for projects is difficult and the AEC does take this in to consideration, however, the information is required under the Code of Practice so make the best estimate and explain why it is not possible to be more accurate (eg. *Trapping has not been carried out in this area before.*) You will need to keep the AEC regularly informed if the numbers exceed your estimate or animals you did not specify in the application. If the project runs into a second/third year you will be expected to be more accurate with your estimates in your Reporting Form. If numbers are increased you will need to submit an Application to modify an approved project.

Click here to enter text.

Species *(common and scientific names)*	Strain (if applicable)	Year	No to be used	No to be killed	Source[6]	Location[7]
		1				Choose an item.
		2				Choose an item.
		3				Choose an item.

Comments:

4. Procedures – Please classify the proposed procedures
(please choose Yes or No. Refer to the detailed endnotes for additional information)
Note: If you are attaching instruments to study animals you will need to complete an AEC Instrumentation Form

1. Field study/capture or study of free-living (including feral) animals [8]	No
2. Marking or tagging [9]	No
3. Behaviour observations	Yes
4. Harvesting of tissues from dead animals	Yes
5. Dissection of dead animals	Yes
6. Anaesthesia with subsequent recovery [10]	No
7. Anaesthesia without subsequent recovery [11]	Yes
8. Surgical procedures [12]	No
9. Use of neuromuscular blockers [13]	No
10. Infection with microbial agents and/or parasites/ testing of toxins [14]	No
11. Implantation/attachment of electrodes, catheters, transmitters, collars, telemetry devices etc. *(If yes, you must submit an Instrumentation form)* [15]	No
12. Blood sampling [16]	Yes
13. Production of antisera [17]	No
14. Forced exercise [18]	No
15. Feeding studies, including diet modification [19]	No
17. Animals with altered genetic make-up *(manipulated, modified, naturally occurring mutation)* [20]	No
18. Administration of pharmaceutical agents [21]	Yes
19. Death as an endpoint (e.g. for toxicological studies) [22]	No
20. If there are other procedures, please detail in the box below:	

5. Detailed Procedures [23]

It is important to provide details (sequentially) on what happens to the animal/s from the time you obtain them until the time the project is completed.

Please ensure that each procedure marked Yes in the table above is described in detail as per the endnotes (provided after the selected procedures). Please include a flow chart or sequence of events table. Your step by step description of what will happen to each animal should include the following:

- Transport, acclimation and conditions of housing and handling;
- Experimental and other procedures, including dose and route of any substance or treatment given and method, volume and frequency of samples collected;
- Surgical and related procedures including dose of anaesthetic, analgesic and tranquillising agents and methods of monitoring their adequacy and side effects;
- The sequence and timing of events from start to finish for individual animals or groups;
- The arrangements for the animal at the completion of the project, including, if applicable, the method of humane killing;
- Include details of the maximum time any individual animal will be held under experimental conditions;
- Include details of where procedures are to be carried out.

Please also refer to the attached checklist to ensure all details have been considered.

Arrival/Housing and general handling

Male Sprague-Dawley rats will be purchased and transported from the UTAS Cambridge Facility 1 week prior to their use and will be housed in groups (2 animals/cage) at the MS2 Animal Facility (6th floor) under standard controlled conditions (22 °C, 12 h light-dark cycle) with continuous access to food and water.
Animals will be monitored before and after every experiment using the Monitoring Checklist 1 & 2 (see Appendix). In days of no scheduled experiment, animals will be monitored once a day using Checklist 1. Some animals will be also monitored after drug exposure and before assessment of nociception levels, using the automated behavioural MCS equipment.

Models/Procedures used in this study

Three models will be used to assess the nociception levels of animals: The "tail-flick test", the "hot-plate" and the "orofacial stimulation test".

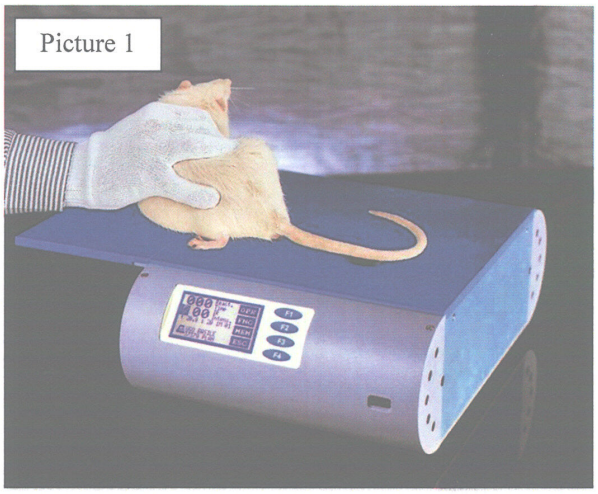
Tail-flick test: The tail-flick test assesses the spinal processes that are used in pain-relief. The animal is placed on a level-surface apparatus **(Picture 1)** with its tail protruding on top of a photoelectric radiant-heat cell. Thermal radiation provokes the animal to withdraw its tail by a brief vigorous movement, a sign of brief discomfort. The apparatus chronometer records the time between the initiation of the beam and the tail retraction. The longer the time, the higher the analgesia experienced by the animal. The exposure time to the radiant heat does not exceed **15sec** (cut-off time). All animals' tail will be monitored for possible tail gnawing after each use, and their temperature is measured by an infra-red thermometer prior and after testing, to confirm no skin inflammation or injury.

Hot-Plate test: The hot-plate apparatus consists of an electrically-heated metallic surface which is kept at a constant temperature of 50 ± 0.5°C, attached to a manual pedal-chronometer **(Picture 2).** The animal is placed on the metallic surface and protected from escaping by a clear plastic border surrounding the plate. The heated plate induces a reflex response to the heat stimulus (paw licking or jumping) which is regarded as a brief discomfort. When this behaviour is presented, the investigator removes the animal from the equipment and records the time. The longer the time, the higher the analgesia experienced by the animal. The exposure time to the plate does not exceed **30sec** (cut-off time). The Hot-Plate test differs from the tail-flick test in that it involves higher order perception of noxious stimuli by the brain.

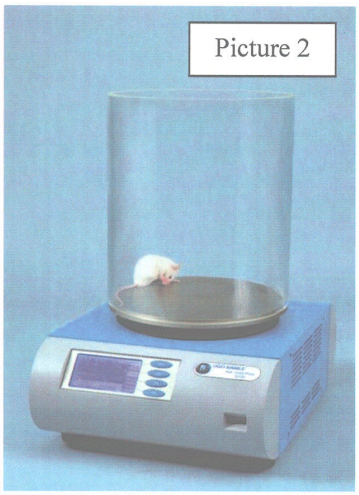
Orofacial Stimulation test: This test measures hypersensitivity to thermal or mechanical stimulation of the trigeminal area of an animal **(Picture 3).** Rats are being fasted for **12 hours** and then placed in a cage with a plastic divider and the orofacial apparatus. Rats will be given **10 mins** to familiarize themselves with their environment. The apparatus has a drinking window for the rat-head to enter and acquire a reward (milk-water) located on the opposing aspect of the drinking window. Nestle Carnation® Sweetened Condensed Milk will be diluted with water to 30% and placed in the cylindrical plastic container with metal nipple drinker in the apparatus. The apparatus also consists of a mechanical or a thermal module **(Picture 4).** An infrared photo-beam is built on the exterior aspect of the drinking window and wired to a computer to automatically detect head accessing the feeding tube. Animals will be subjected to training for 10munutes after the fasting period, where they will be allowed to drink the

reward without the use of the modules, and then return to their cage. After a new **12 hour** fasting period, animals will enter the apparatus and depending on the type of the experiment, they will be subjected to either no stimulus during the testing period, to a mechanical, or to a thermal stimulus when it attempted to poke its head through the drinking window for **10mins**. The orofacial testing provides assessment of nociception levels that are based on processes analysed by the brain, without the need of the spinal cord (References included in Appendix 6).

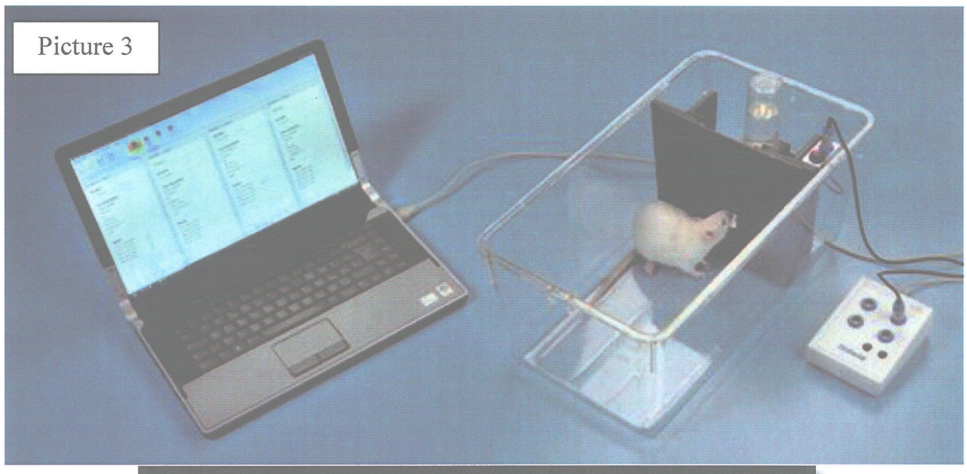
Picture 1



Picture 2



Picture 3



Picture 4

The **thermal stimulator** relies on a copper tubing loop and a circulating water bath, whose temperature can be adjusted from ambient to 70°C, to reach hot nociceptive thresholds. Chin inserts are included to test animals of different size.

The **mechanical stimulator** relies on thin wires attached to a mounting plate. The system comes with several plates, each with a different number of wires in order to apply different force levels to the animal vibrissal pad.

Experiment 1: effect of weight on morphine analgesia

We need to find out whether the analgesia produced by increased dose of morphine is predicted by the increase in the weight of the animals. We will be testing the nociception levels of six obese rats (~1kg) feeded with a high-fat diet, using the tail-flick equipment described above.

- **Procedure**: as described above for tail-flick
- **Duration**: testing time of 1min, for every 30mins during a **2 hour** experiment during one day.
- **Animal numbers: 9**
- **Groups**:
  Control Group = **3** rats given saline, IV
  Treatment Group 1 = **3** rats given morphine, one IV injection of 10mg/kg.
  Treatment Group 2 = **3** rats given morphine, one IV injection of 30mg/kg
- **Animal fate**: all animals in this experiment will be killed according to the described killing method described (section 13.2).

Experiment 2: acute administration of UTAS drugs

We will be administering the UTAS drugs to the animals by acute SC injection.

- **Procedure**: animals will be placed in the tail-flick equipment to assess their nociception levels, prior and after drug administration (1 SC injection/animal, 40-100µl 10nmol), as described above.
- **Duration**: testing time of 1min, for every 30mins during a **2 hour** experiment during one day.
- **Animal numbers: 45**
- **Groups**:
  Treatment Group 1 = **3** animals for each of **6 UTCP** drugs, 10nmol SC = total **18** animals
  Treatment Group 2 = **3** animals for each of **6 UTCP** drugs, 50nmol SC = total **18** animals
  Positive Control Croup = **3** animals with **morphine** 10nmol SC
  Negative Control Group = **3** animals given **saline** SC
  Backup = **3** animal
- **Animal fate**: all animals in this experiment will be killed according to the described killing method described (section 13.2).

Experiment 3: acute administration of the two highest efficacy UTAS drugs tested

We will choose the 2 best UTAS drugs from those tested in Experiment 2, in order to produce a dose-response analysis of analgesia.

- **Procedure**: animals will be placed in the tail-flick equipment and hot-plate equipment to assess their nociception levels, prior and after drug administration (1 SC injection/animal, 40-100µl, range of 6 increased doses).
- **Duration**: testing time of 1min, for every 30mins during a **2 hour** experiment during one day.
- **Animal numbers: 25**
- **Groups**:
  Treatment Group = **3** animals for **1 UTCP** drug, in 6 increased doses, SC = total **18** animals
  Positive Control Croup = **3** animals with **morphine** 10nmol SC
  Negative Control Group = **3** animals given **saline** SC
  Backup = **1** animal
- **Animal fate**: all animals in this experiment will be killed according to the described killing method described (section 13.2).

Experiment 4: Chronic administration

The purpose of this experiment is to determine whether the UTAS candidate drug produces tolerance compared to morphine.

- **Procedure**: animals will be placed in the tail-flick equipment and hot-plate equipment to assess their nociception levels, prior and after drug administration (1 SC injection/animal/day, 40-100µl, three doses determined from Experiment 3) for a duration of 8 days.
- **Duration**: animals tested for 2 hours per day, for **8 days**

- **Animal numbers: 25**
- **Groups**:
  Treatment Group = **5** animals for **1 UTCP** drug, for each of 3 doses, SC = total **15** animals
  Positive Control Croup-1 = **3** animals with **morphine** 10nmol SC
  Positive Control Group-2 = **3** animals with **morphine** 10nmol SC + **naltrindole** 10nmol SC
  Negative Control Group = **3** animals given **saline** SC
  Backup = **1** animal
- **Animal fate**: all animals in this experiment will be killed according to the described killing method described (section 13.2).

Experiment 5: Acute administration of three routes of administration

The purpose of this experiment is to determine whether the UTAS candidate drug can produce analgesia in three different routes of administration

- **Procedure**: animals will be placed in the tail-flick equipment and hot-plate equipment to assess their nociception levels, prior and after acute drug administration (1 SC injection/animal, 40-100µl, maximum dose determined from Experiment 3).
- **Duration**: animals tested for 2 hours during one day
- **Animal numbers: 24**
- **Groups**:
  Treatment SC Group = **5** animals for **1 UTCP** drug, for 1 max dose, subcutaneous
  Treatment IP Group = **5** animals for **1 UTCP** drug, for 1 max dose, intraperitoneal
  Treatment PO Group = **5** animals for **1 UTCP** drug, for 1 max dose, oral
  Positive Control Croup = **3** animals with **morphine** 10nmol SC
  Negative Control Group = **3** animals given **saline** SC
  Backup = **3** animal
- **Animal fate**: all animals in this experiment will be killed according to the described killing method described (section 13.2).

Experiment 6: Orofacial stimulation test

The purpose of this experiment is to determine whether the UTAS candidate drug can produce analgesia in an orofacial test, as described in the procedures analytically above.

- **Procedure**: animals will be fasted for 12 hours, trained in the orofacial equipment for 10mins, then returned in their cages. Next day the animals will be fasted for 12 hours, and tested in the orofacial equipment for 10mins.
- **Duration**: **2** days
- **Animal numbers: 12**
- **Groups**:
  Treatment Group = **5** animals for **1 UTCP** drug, for 1 max dose, one SC injection
  Positive Control Croup = **3** animals with **morphine** 10nmol SC
  Negative Control Group = **3** animals given **saline** SC
  Backup = **1** animal
- **Animal fate**: all animals in this experiment will be killed according to the described killing method above (p8).

Experiment 7: Pharmacokinetics

The purpose of this experiment is to determine how the body handles the UTAS drug candidate and where does the drug go to after administration.

- **Procedure**: animals will be placed in the tail-flick equipment to assess their nociception levels, prior and after acute drug administration (2 administrations/day, two days, maximum dose determined from Experiment 3). The appropriate rout of administration will be determined from Experiment 5.
- **Duration**: **2** days
- **Animal numbers: 12**

- **Groups**:
  Treatment Group = **5** animals for **1 UTCP** drug, 2 administrations/day, two days
  Positive Control Croup = **3** animals with **morphine** 2 administrations/day, two days
  Negative Control Group = **3** animals given **saline** 2 administrations/day, two days
  Backup = **1** animal
- **Animal fate**: all animals in this experiment will be killed according to the described killing method described (section 13.2).

Note:

All researchers included in this application and carry out any of the above procedures will be trained and assessed by the AWO.

6. Monitoring by Investigators – Please detail below how the well-being of animals will be assessed throughout the project.

6.1 Provide details of the location, the method and frequency of day-to-day monitoring of animals before, during and after procedures (Please attach to your application a copy of the check sheet you will use to record this information)

Before Procedures:	Animals will be monitored in their cages daily, using the Rat Monitoring Checklist-1 shown in the Appendix. If animals do not undergo any procedure at a given day, those rats will still be monitored once/day using the same list. Monitoring will be completed by the associate investigator. The checklist includes all relevant guidance and action points.

During Procedures:	Animals will be monitoring continuously during the procedures

After Procedures:	Exactly the same as Before Procedures above. At the days of experiments, animals will be monitored using the checklist twice/day. At the days of no experiments, animals will be monitored once/day only.

6.2. What clinical, behavioural or other signs will be used to indicate that intervention is needed to alleviate an animal's pain or suffering? What action will be taken if these indicators are reached?
(Please attach a copy of the check sheet you will use to record this information to your application)

In the unlikely event of an animal experiencing pain or suffering for any reason, there are specific clinical & behavioural signs that the investigator will look for and record, before, during and after the experiments (this regular check is important, since the study involves behavioural testing). These signs include increased vocalization, behaviour of isolation, significant changes in weight, reduced activity and others. These signs are categorized in three distinct categories based on their significance and severity. A complete list of the signs or symptoms is shown in the check lists available in the Appendix (p.26 and 27), as well as the specific actions that need to be taken for each of those (p.28).

6.3 Who will be responsible for the management of veterinary and other emergencies and how will it be ensured that researchers are aware of the person's location and know how to contact them quickly?

The **responsible investigator** (Dr Nikolas Dietis, Pharmacy, tel 6226 1003, mobile 0467981698) will be response for the management of emergencies and all actions taken, named as the "Supervisor" in the interventions listed in the Monitoring Checklist.
The contact info of the investigator will be located both in the equipment manuals in the behavioural rooms, but also in the Monitoring Checklists.

7 Location

7.1 If the study is taking place on UTAS premises, please state exactly where.

Menzies MS2 Level 6 animal facility. All experiments will take place in the Rat Behavioural Room. Decapitation will be carried out in Room 625 at the MSP as advised by the Animal Services Manager.

7.2 If the study is not taking place on UTAS premises, please state exactly where.

N/A

8. Replacement (Code of Practice 1.18 – 1.20)

Please provide an explanation as to why animals are needed for the project. Please include:

- a list of any potential alternatives to animal use;
- whether any of these alternatives are being used in this project, and if not;
- why alternatives are unsuitable for this project.

This project aims to study analgesia. Only higher organisms (in particularly mammals) have the capacity to present analgesic behaviour. Our equipment allows us to study pain-like behaviours only in rats. Our previous experiments with our drug candidates have also used rats and we aim to combine the animal data of different studies, therefore the same species has to be used across studies.

9. Reduction (Code of Practice 1.21 – 1.27)

9.1 On the basis of experimental design and statistical analysis explain why this number of animals will be required. Include details of your estimation of animals required to cover mortalities due to events outside your research i.e. death during transportation etc.
This must be the minimum number of animals that is necessary in order to obtain a valid result.

Previous studies have shown that the statistical power for acute (short-term) experiments requires a minimum sample size of n=3 or n=5 for some groups, depending on the experiment, in order to give reproducible and accurate results.

9.2 Is there an opportunity for sharing of tissue or animals used in the project?

No, all animals will be killed after the end of the experiments, due to the need of tissue analysis

10. Refinement (Code of Practice 1.28 – 1.30)

What measures will be taken to reduce the impact of the investigation on the animals to be used?
Refinement might be achieved in a number of areas e.g. research design, procedures, housing conditions, animal handling.

The actions to be taken in order to keep the impact of the investigation on the animals to minimum are described in detail the Procedures, including housing conditions and general animal handling measures before, during and after the experiments.

11. Animal Welfare Impact

11.1 What degree of pain, suffering or distress will be experienced by the animals during the project and how will this be measured?

The project will use various stimuli that aim to cause some degree of discomfort, for the purpose of the study. The analgesic equipment used (tail-flick, hot-plate and orofacial tests) will cause a very brief and mild discomfort (few seconds), where the animal is free to react to (withdrawal of tail or licking of the paw). Once these reaction signs are observed, the discomfort will cease as the animal is removed from the experimental setting.
No pain or distress is expected after drug exposure before the killing of animals, since all agents used are expected to be analgesics.

11.2 Please justify the level of pain, suffering or distress (Code of Practice 2.7.4 v)
NB: Particular justification must be given for potentially severe or ethically contentious procedures, e.g:

- Unrelieved pain and distress including where the planned end-points will allow severe adverse effects to occur (see Code of Practice ;
- Death as the end point;
- Prolonged restraint or confinement;
- Production of monoclonal antibodies by the ascites method (Code of Practice 3.3.31) and
- The use of non-human primates (Code of Practice 2.4.8 iv).

N/A

11.3 What factors affecting the wellbeing of animals will lead to premature termination of the experiment, and how will this be assessed, e.g.

- Tumour size;
- Maximum weight loss;
- Excess stress of the animals.

It is unlikely that the planned experiments will give rise to issues that might lead to exclusion of animals from the experiment. However, there are some indications that when observed will alert the investigators to initiate intervention which could lead to the termination of the participation of this animal from the rest of the experiments. These indications are listed in the Montitoring Checklists as Category 2 and 3 (see Appendix).

12. Repeated use of Animals (Code of Practice 1.22, 1.23 and 2.3.15)

Have the animals been used in a previous project? Yes / **No**
If yes the AEC will need to determine the total impact on the welfare of the animals' involvement in multiple projects. Please provide details, particularly with regard to the following:
Briefly describe the procedures that each animal or group of animals has been subjected to in the previous project. Highlight any pain, distress or potential cumulative effects of previous procedures. Also describe the time the animal has been used in previous project/s, the recovery period between projects and the total time the animal has been used.

13. Fate of the Animals

13.1 What will happen to the animals when you have finished with them?

All animals will be killed for the purpose of the study.
Please refer to the Procedures under the titles "Fate of animals" for more details.

13.2 If animals are to be killed, please explain how this will be done?

All animals in Phase 3 & 4 will be killed by the following method:

This method will involve the induction of anaesthesia by inhaled isoflurane, before the final decapitation of the animal immediately after anaesthesia induction.
We therefore propose to apply the following procedure for gaseous anaesthesia induction:

- The anaesthetic machine set up will be checked prior to use to ensure it is in working order. The

isoflurane level of the machine will be checked to ensure adequate levels. The oxygen tap that supplies the anaesthetic machine will then be turned on.

- The induction chamber will be checked to ensure it is attached to the anaesthetic machine, and to ensure it is adequately clean.
- The oxygen flow will be turned on (flow rate of 500-1000ml/min).
- The rat will be placed into the induction chamber and the lid screwed down to seal the chamber.
- Isoflurane will then be turned on (to 5% flow)
- The rat will be monitored continuously until it has adequately loss consciousness.
- The rat will be checked to ensure it is recumbent and there is no righting reflex and no withdrawl reflex when pinching the toes.
- The isoflurane and oxygen will be turned off and the rat removed from the induction chamber.
- The rat will immediately be moved to the decapitator which is placed on an adjacent bench.
- The rat will be monitored until decapitation to ensure it is not rousing from anaesthesia (as isoflurane is very short acting gaseous anaesthesia).
- The rat will then be decapitated.

In the event that the rat is showing signs of regaining consciousness, it will be placed back into the induction chamber and the procedure will be repeated to ensure loss of consciousness.
The isoflurane-induced anaesthesia is quickly becoming the standard method of general anaesthesia for rats and mice used in biomedical research. It holds many advantages over injectable agents, CO2 or live-decapitation, such as: minimal animal handling, margin of safety, ease of anaesthetic control, low cost of anaesthetic agent, no controlled drugs, among others.
Full training of the above proposed method of killing will be provided by the Animal Welfare Officer, after which will be performed by the responsible investigator.

13.3 Will any animals be killed in the presence of other animals? Please justify.

A decapitator will be used opposite the procedure bubbles near the sink and no other animals will be present at that end point.

13.4 Who will kill the animals?

The Responsible investigator. The associate investigator will assist only on cleaning and preparation of the guillotine.

14. Risks

Please specify any special risks to other animals or humans arising from the project.

No anticipated risks. Contingency plans for the killing procedure are described in Section 13.2. All precautions taken in this study are described in detail in Appendix 1.

15. Funding

The funds for all the experiments covered in this project are fully covered by the Division of Pharmacy

15.1 If you have submitted a grant application, please provide the University of Tasmania Grant Reference Number

N/A

15.3 If your application for funding is unsuccessful, how do you intend to fund the project?

N/A

16. References

Please provide full details of any references referred to in the text of this application.

1. Dietis N, Guerrini R, Calo G, Salvadori S, Rowbotham DJ, Lambert DG. Simultaneous targeting of multiple opioid receptors: a strategy to improve side-effect profile. British Journal of Anaesthesia. 2009; 103: 38-49.

2. Dietis N, McDonald J, Molinari S, Calo G, et al. Pharmacological characterisation of the bifunctional opioid ligand H-Dmt-Tic-Gly-NH-Bzl (UFP-505). British Journal of Anaesthesia. 2012; 108(2): 262-70.

3. Dietis N, Ruggieri V, Filaferro M, Novi C, et al. Antinociceptive effects of the bifunctional opioid UFP-505 in rats. In Proceedings of the Anaesthetic Research Society Meeting. British Journal of Anaesthesia. 2011; 107(5): 826–38P

4. Dietis N, Niwa H, Tose R, McDonald J, Vitale G, et al. Aversion of tolerance by the bifunctional opioid ligand H-Dmt-Tic-Gly-NH-Bzl (UFP-505); in vivo and in vitro characterisation. (Manuscript currently under construction).

17. Personnel

On the following tables, list the qualifications, experience and ethics training of all personnel in this project who will be involved with live animals. Detail their experience with the species being used as well as the procedures being undertaken. In particular, when surgical or other procedures are involved, the Committee will look closely at investigators' experience before giving approval.

Please ensure that all investigators read the following declaration:

- I am familiar and will comply with all relevant Commonwealth and State or Territory legislation and the requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th edition 2013.
- I accept that I have a responsibility to ensure that the investigations and procedures proposed in this form are conducted fully within the conditions laid down by the Code and I undertake not to use any animals or carry out any procedures which have not been approved by the Committee.
- I accept that the responsible investigator has ultimate responsibility for the project.

All personnel involved in the project MUST sign this document before it can be submitted to the AEC

Responsible Investigator

Title:	Doctor	First Name:	Nikolaos
Surname:	Dietis	Position:	Lecturer
Email:	Nikolaos.Dietis@utas.edu.au	Telephone:	6226 1003
Telephone (Mobile)	0467 9816 98	Fax:	Click here to enter text.

When did you last attend a UTAS Ethics Training Seminar? (Approximate date): Click here to enter a date.

I have never attended a UTAS Ethics Training Seminar Yes/No

If you have not attended a UTAS Ethics Training Seminar within the last three years you MUST attend the next available session details of which are available on the Animal Ethics Website.

Signature: ·

Date: 25/02/2014

Nominee [24]

Title:	Associate Professor	First Name:	Nuri
Surname:	Guven	Position:	Senior Research Fellow
Email:	Nuri.Guven@utas.edu.au	Telephone:	6226 1715
Telephone (Mobile)	0470 380 090	Fax:	Click here to enter text.
School/Discipline:	School of Pharmacy	Internal Box No:	26

When did you last attend an Ethics Training Seminar? (Approximate date): Click here to enter a date.

I have never attended a UTAS Ethics Training Seminar Yes/No

If you have not attended a UTAS Ethics Training Seminar within the last three years you MUST attend the next available session details of which are available on the Animal Ethics Website.

Signature: ····

Date: 25/02/2014

Associate Investigator [25]

Title:	Mr	First Name:	Alok
Surname:	Paul	Position:	PhD student
Email:	Alok.Paul@utas.edu.au	Telephone:	Click here to enter text.
Telephone (Mobile)	0470 629154	Fax:	Click here to enter text.
School/Discipline:	Pharmacy	Internal Box No:	Click here to enter text.

When did you last attend an Ethics Training Seminar? (Approximate date): 2014

I have never attended a UTAS Ethics Training Seminar Yes/**No**

If you have not attended a UTAS Ethics Training Seminar within the last three years you MUST attend the next available session details of which are available on the Animal Ethics Website.

Signature:

Date: 25/02/2014

18. Competency Assessment

Note: Pursuant to the requirements of the Regulations to the Veterinary Surgeons Act 2012, persons conducting procedures defined as veterinary services[26] for the purpose of research, including sedation, anaesthesia and euthanasia, unless defined as a prescribed services,[27] are required to be assessed as competent in the procedure by a registered vet before commencing, and thereafter competence must be assessed annually. The University of Tasmania requires that the assessment of competency be made by the Institution's Animal Welfare Officer and approved by the Animal Ethics Committee.

Please provide the following information:

Technique/ Procedure Please list each separately e.g handling & restraint, injection (route), trapping (method), sampling (what & how)	Species (in which technique is proposed)	Name of person/s performing procedures	Level of Experience (no. of times procedure performed) None / Limited / High (<5) (5-20) (>20) If inexperienced provide name of proposed trainer	Year last performed
General handling	SP rat	Dr N Dietis Mr Alok Paul	High High	2014 2014
Restraint	SP rat	Dr N Dietis Mr Alok Paul	High High	2014 2014
Injection (SC)	SP rat	Dr N Dietis Mr Alok Paul	Limited None	2014 2014
Decapitation	SP rat	Dr N Dietis	High	2014
Tissue removal	SP rat	Dr N Dietis Mr Alok Paul	High Medium	2014 2014
Behaviour test(pain) - hot plate	SP rat	Dr N Dietis Mr Alok Paul	High Medium	2014 2014
Behaviour test(pain)- infra red tail flick	SP rat	Dr N Dietis Mr Alok Paul	High Medium	2014 2014
Behaviour test(pain) - orofacial stimulation	SP rat	Dr N Dietis Mr Alok Paul	High Medium	2014 2014

19. Other Authorities Involved

19.1 UTAS Cambridge Farm Facility (CFF) Yes

If the CFF is involved the Animal Services Manager, Mr Paul Scowen, must sign this document BEFORE you submit it to the AEC

Declaration by CFF Curator

I have read this document and approve of the involvement of the CFF.

Name of CFF Curator: Paul Scowen

Signature:

Date: 4/03/2014

19.2 Has Institutional Biosafety Committee (IBC) approval been obtained?

Under the Australian Code of Practice all projects involving the genetic modification of animals must be conducted in accordance with the requirements and guidelines of the UTAS Institutional Biosafety Committee. When animals with an altered genetic makeup are to be included in the study, the IBC approval number must be provided.

IBC Consulted **N/A**

IBC Approval Obtained **N/A**

- If approval has been obtained please append a copy of the approval and type the number below. If not, you *must* obtain and submit a copy of the approval to the AEC on receipt. Approval of a project may be suspended by the AEC if this is not provided

Approval Number/Comments:

19.3 External Authorities

Are there any external authorities involved in the project? No

If Yes, please provide details of the relevant authority (including approval numbers and dates). Please append copies of approval notifications.

Click here to enter text.

Government departments? (re. approval of joint projects)[28] **No**

Click here to enter text.

Government departments? (re. provision of permits) **No**

Click here to enter text.

Other Universities? **No**

Click here to enter text.

Any other external authority e.g. industry partners, other institutions, other persons **No**

Click here to enter text.

Other Animal Ethics Committee No

Click here to enter text.

Declaration by External Authority

If any external authorities are responsible for the daily care of animals a signature must be provided.

- I confirm that I will be responsible for the daily care of the animals for the research period.

Name: Click here to enter text.

Signature: .....

Department/Organisation: Click here to enter text.

Date: Click here to enter a date.

20. Use of Animals in Queensland

Will you be using animals for scientific purposes in Queensland? (Please choose Yes or No) **No**

If 'YES' you will need to sign the following declaration and forward the approved initial application form and University of Tasmania AEC approval memo to the Queensland Department of Primary Industries Animal Welfare Unit before animal use starts.

Declaration by Responsible Investigator

I confirm that as a member of staff or student of the University of Tasmania, I am registered by the Queensland Government Department of Primary Industries as a person who can use animals for scientific purposes.

Signature: .....

Date: Click here to enter a date.

Registration Certificate Number: 0007
Registered Name: University of Tasmania

CHECKLIST

Has the following information been provided?

Anaesthesia
Fasting
Induction - drug, dose, route
Maintenance - drug, dose, route
Methods of monitoring anaesthesia and recovery
Additional support during anaesthesia and recovery
(eg heat, intravenous fluids)
Location of induction and recovery areas
Restraint
Expertise of personnel

Behaviour modification
Stimulus (type, duration, frequency)

Blood/body fluid collection
Volume
Route
Frequency
Anaesthesia or analgesia
Restraint
Animal monitoring (methods, frequency)

Diet/water modifications
Type
Amount
Effects
Measurement of intake
Animal monitoring

Drug treatments
Substance
Volume/dose
Route
Frequency/total number per animals
Local and systemic effects
Anaesthesia or analgesia
Possible side effects
Restraint
Expertise of personnel

Euthanasia
Method

Surgery
Anaesthesia
Location of pre-operative preparation area
Pre-operative preparation
Surgical procedure (site, technique)
Sterile technique (instruments, drapes, surgeon)
Location of and housing in post-operative recovery area
Post-operative management
Post-operative monitoring (methods, frequency, duration)
Use of analgesics (type, dose, route, frequency,
means of determining necessity for use)
Expertise of personnel

Tumour/neoplasia induction
Method
Site
Endpoint
Animal monitoring (methods, frequency)

Toxicology
Substance
Volume/dose
Route
Frequency of treatment/total no per animals
Local and systemic effects
Anaesthesia or analgesia
Restraint
Animal monitoring (methods, frequency)
Endpoint/duration

Wildlife studies
Location
Methods
Capture methods
Handling/restraint
Housing
Monitoring
Release
Effects on population

Location (where procedure will be performed)
Expertise of personnel

Genetic manipulation
Methods
Potential effects

Teaching
Source of animals
Housing
Duration held
Method of disposal

In-vitro studies
Source of animals
Duration held
Euthanasia

Transport
Type
Duration
Confinement
Numbers of animals
Airconditioning
Housing
Location
Isolation
Group housing (stocking rates, sexes)
Shelter
Bedding
Hiding areas
Environmental enrichment
Duration held
Conditioning period

ATTACHMENT A: TEACHING ACTIVITIES

Please refer to Section 4 of the Code when completing this section.
It must be completed for all teaching activities (as indicated in Section A1 of this form).

1. What is the maximum number of students to be supervised by each teacher?

Click here to enter text.

2. Please specify the number of animals to be used by each student

Click here to enter text.

3. What is the maximum number of times each animal will be used throughout this project?

Click here to enter text.

4. How will the attainment of educational objectives be assessed?

Click here to enter text.

ATTACHMENT B: DECLARATION FOR STUDENTS

Declaration for students involved in a teaching project involving live animals, and not named as an associate investigators

(This sheet may be printed to allow for signatures, scanned and submitted with your application)

Animal Ethics Committee approval number: Click to Enter Text

Responsible investigator: Click to Enter Text

Please sign to certify the following:

- I am familiar and will comply with all relevant Commonwealth and State or Territory legislation and the requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th edition (2004);
- I accept that I have a responsibility to ensure that the investigations and procedures proposed in this form are conducted fully within the conditions laid down by the Code;
- I undertake not to use any animals or carry out any procedures which have not been approved by the Committee;
- I accept that the responsible investigator has ultimate responsibility for the project.

FULL NAME	SIGNATURE	DATE

Endnotes and further information relating to the form:

[1] Title: The title should be concise but specific. Please include species name - both common and scientific.

[2] Responsible (Chief) investigator: The Committee accepts the *Australian Code of Practice* policy that one person is ultimately responsible for the project. Accordingly, joint responsible investigators must not be named. The person named as the Responsible Investigator (RI) must be a member of the University staff on an academic or equivalent grade. Supervisors will therefore normally be the responsible investigators for projects involving honours and postgraduate students. The RI should at all times be aware of experimentation being conducted by members of a team.

[3] Person who has experience as a Responsible Investigator and is qualified to take responsibility in the absence of the Responsible Investigator above.

[4] Source: please detail where animals will be obtained. If the animals are from a native captive colony, please give the barcodes of all animals that will be used. If the animals are to be wild caught, you will need to obtain approval from the appropriate government department and forward a copy of the approval to the Executive Officer. You should refer to the *Parks and Wildlife Code of Practice for the Keeping of Animals in Tasmania (September 2002)* (please follow the link on the Animal Ethics webpage). You should also append details of procedures to be followed in the event of any by-catch, including record keeping to be undertaken. If animals are to be released back into the wild you will need to provide detailed information on the release process.

[5] 'Location' refers to the state/country in which the animals were used. Please select one number from the list below. If the same species has been used in 2 or more different locations, please select number 10 and provide a breakdown of the **number of animals used in each location** in the 'Additional information' box below the table.

1. Tasmania
2. Victoria
3. New South Wales
4. Queensland
5. Northern Territory
6. South Australia
7. Western Australia
8. Australian Capital Territory
9. Overseas
10. Species used in more than one state/country (please provide a breakdown of the number of animals used in each location in the 'Additional Information' box below the table).

[6] Source: please detail where animals will be obtained. If the animals are from a native captive colony, please give the barcodes of all animals that will be used. If the animals are to be wild caught, you will need to obtain approval from the appropriate government department and forward a copy of the approval to the Executive Officer. You should refer to the *Parks and Wildlife Code of Practice for the Keeping of Animals in Tasmania (September 2002)* (please follow the link on the Animal Ethics webpage). You should also append details of procedures to be followed in the event of any by-catch, including record keeping to be undertaken. If animals are to be released back into the wild you will need to provide detailed information on the release process.

[7] 'Location' refers to the state/country in which the animals were used. Please select one number from the list below. If the same species has been used in 2 or more different locations, please select number 10 and provide a breakdown of the **number of animals used in each location** in the 'Additional information' box below the table.

11. Tasmania
12. Victoria
13. New South Wales
14. Queensland
15. Northern Territory
16. South Australia
17. Western Australia
18. Australian Capital Territory
19. Overseas
20. Species used in more than one state/country (please provide a breakdown of the number of animals used in each location in the 'Additional Information' box below the table).

[8] You will need to include (with references) under 'Detailed Procedures' details on permits required for this study and whether they have been obtained/applied for. You should refer to the *Parks and Wildlife Code of Practice for the Keeping of Animals in Tasmania (September 2002)* (please follow the link on the Animal Ethics webpage). Detail the target species of the study and

whether they are classified as rare/endangered. If you plan to capture the animals justify why this is necessary. Outline capture techniques, tools (including trap sizes, baits etc), timeframes (including frequencies of monitoring traps), release details (including times of release with references). Outline what emergency plans are in place in the case of unexpected incidents (eg weather conditions, illness etc) including provisions for animals captured with/without young and lactating animals. How will animals be handled/restrained? If transportation is required, describe how this will occur and included details on monitoring of animals. How will individual animals be identified? If tracking devices will be implanted/attached, you will need to give detailed information on the size, weight and method of attachment and removal of the devices, and monitoring of the animals after implantation/attachment.

[9] You will need to include (with references) under 'Detailed Procedures' details of size, colour, position, etc and method of marking and tagging. Outline why these methods are being used. If these methods have been used previously, please consider and outline any way the marking method could be refined.

[10] You will need to include (with references) under 'Detailed Procedures' the type of anaesthesia and dose rates and administration routes to be used, methods of monitoring the level of anaesthesia, details of analgesics to be used (including dose rates, frequencies and administration routes), provide details of post operative care and monitoring during anaesthesia recovery and longer term post procedure, and contingency plans in the event of unexpected outcomes.

[11] You will need to include (with references) under 'Detailed Procedures' the anaesthetic, the dose rate, administration route (s), who will conduct the anaesthesia and euthanasia, assessment methods for monitoring the level of anaesthesia, the method of disposal of euthanased animals and contingency plans in the event of unexpected outcomes.

[12] You will need to include (with references) under 'Detailed Procedures' detailed information about the surgery, eg duration, personnel, location, aseptic and general surgical techniques to be employed, and possible complications associated with surgical procedures and protocols for management of complications.

[13] Please note: neuromuscular blocking agents must not be used without adequate general anaesthesia or an appropriate surgical procedure that eliminates sensory awareness. You will need to include (with references) under 'Detailed Procedures' reasons why a neuromuscular blocking agent is required and what physiological parameters will be monitored to assess adequacy of anaesthesia.

[14] You will need to include (with references) under 'Detailed Procedures' details on precisely what agents/parasites will be used in this study and what effect the agent(s)/parasite(s) will have on the subject animals. You should detail whether appropriate Institutional/State approval has been issued for the use of the agent(s)/parasite(s) and whether personnel involved with the project are aware of its use and effects, including animal house staff and associate investigators. Where will the infection take place and where will infected animals be housed? What level of microbiological containment is required and is this available in the designated experimental/housing sites?

[15] You will need to include (with references) under 'Detailed Procedures' details of the size and position of implants etc, surgical or other procedures, post implantation/attachment wound management, timeframes, and plans for removal. Please include what the most likely negative sequelae to implantation etc will be and how these will be managed. If the transmitters etc are to be removed, please detail how the animals will be monitored after removal (eg for wound breakdown etc).

[16] You will need to include (with references) under 'Detailed Procedures' details on who will collect the blood, the anatomical location of the sampling, whether the animal will be sedated or anaesthetised (if so, how), the volume of blood to be taken (and the percentage this constitutes of the animal's circulating blood), the frequency of blood collection, measures of monitoring the animals and contingency plans in the event of unexpected outcomes.

[17] You will need to include (with references) under 'Detailed Procedures' details of antigen and adjuvant including volume, routes and programme of administration. Please detail when the animals will be bled for sampling and for final collection of sera.

[18] You will need to include (with references) under 'Detailed Procedures' what methods will be used to force exercise. Please include the following: What parameters will be measured to determine levels of exertion? What parameters are known (eg max speed, duration of max speed, ability to endure continued exercise) for the animal you are working with? You should detail what behaviour the animals would exhibit if excessively stressed, and include the criteria that will be used to determine that the animals are becoming excessively stressed leading to the termination of forced exercise.

[19] You will need to include (with references) under 'Detailed Procedures' details of the normal maintenance requirements for the animals, growth requirements if animals are immature etc. How will the trial diets vary from the 'normal' diet? What are the possible negative outcomes from diet restriction and/or modification? What parameters will be used to measure the well being of the animal? What endpoints will be used in order to determine when or if an animal is not coping with the trial or diet modification and should be removed from the trial?

[20] You will need to include (with references) under 'Detailed Procedures' the type and strain of genetic alteration; justify the use of such animals (in lay terms) and describe any expected impacts of the alteration in the genotype on the welfare of the animal. Please outline any problems associated with the housing or use of these animals and what level of containment is required. Institutional Biosafety Committee (IBC) approval **MUST** be sought for all projects that involve biohazards to other humans or animals and for all projects that involve the genetic modification of animals. Biohazards are infectious agents or hazardous biological materials that present a risk or potential risk to the health of humans, animals or the environment. The risk can be direct through infection or indirect through damage to the environment.

[21] "You will need to include (with references) nder 'Detailed Procedures' a list of any analgesics or other medically related pharmaceutical agents that the animals may receive. Please include the identification, dose, and route of administration of these agents. Please identify any scheduled drugs and provide details of the source of these.

[22] Death as an endpoint: When the death of an animal is the deliberate measure used for evaluating biological or chemical processes, responses or effects. That is, where the investigator or teacher
with not intervene to kill the animal humanely before death occurs in the course of a scientific activity.

[23] Detailed procedures: Give a clear step-by-step description of the design of the investigation and all procedures to be carried out on each group of animals (including controls) from the beginning to the end. The duration of all procedures and time sequence must also be clear. Flow charts, diagrams and/or tables may be helpful. Details should include treatment substances, dose rates, routes of administration, anaesthetic and analgesic regimes, method of euthanasia, identified end points, etc. if applicable. It must be clear which investigators will carry out particular procedures (refer to the checklist at the end of this form to check that all details have been considered), and WHERE the procedures will be performed. The Committee must be satisfied that pain or distress to animals is avoided or, if this is not possible, minimised. Applications will be examined carefully in this respect. Investigators must give detailed and specific information about any procedures that have the potential to cause pain or distress.
[24] Nominee: Normally one of the associate investigators will be named as the nominee. The nominee must be able to act as the responsible investigator when necessary and therefore must be a member of the University (or in the case of AMC applications, an AMC staff member) , staff on an academic or equivalent grade. In the event of the nominee not being an associate investigator he/she must nonetheless have thoroughly read the proposal and be fully conversant with the project. The nominee will assume the responsibilities of the Responsible Investigator during any absence of the CI.

[25] Associate investigators: Any other persons who will perform procedures on live animals as part of a research project or teaching exercise must be named as associate investigators and must sign on the application form to confirm that they are familiar with the *Australian Code of Practice*. 'Procedures' include benign procedures/manipulations such as diet manipulation. For teaching practicals involving a number of undergraduate students, details must be provided on the Student Declaration Form found under the Animal Ethics Heading at: http://www.utas.edu.au/research/divisional-resources/forms

[26] A "**Veterinary Service**" means any service that forms part of the practice of veterinary surgery and includes but is not limited to:

i. the examination of, or attendance on, any animal for the purposes of diagnosing the physiological or pathological condition of the animal, including testing or imaging for diagnostic purposes;

ii. giving advice based on a diagnosis referred to in paragraph (i), including prescribing treatment, drugs, medications or medical appliances;

iii. performing medical or physical treatment of animals;

iv. performing surgical procedures on animals;

v. administering an anaesthetic to any animal;

vi. conducting pregnancy testing of any animal;

vii. carrying out, by manual operation or use of instrumental appliances, any procedure on an animal for artificial breeding purposes; or

but does not include any prescribed services

[27] Exempt Procedures: For the purposes of the definition of veterinary services in section 3(1) of the Act, the following are prescribed services:

(a) tail docking of lambs that are 6 months old or less;

(b) mulesing of lambs that are 6 months old or less;

(c) deworming that does not involve oesophageal intubation (stomach tube);

(d) castration of oxen, sheep or goats that are 6 months old or less;

(e) castration of pigs that are 2 months old or less;

(f) sexing chickens;

(g) debeaking chickens;

(h) removal of horn, horn core and associated skin in goats, or oxen or sheep, that are 6 months old or less;

(i) removal of horn or antler buds from any species before the formation of horn or pedicels (antler bases);

(j) removal or partial removal of antlers or horns from any species, provided that no vascular or other living tissue is removed with the antler or horn;

(k) filing or rasping of horse teeth without the use of power tools;

(l) shoeing of horses;

(m) non-invasive massage;

(n) collection of faecal samples;

(o) collection of milk samples;

(p) collection of blood samples at the direction of a registered veterinary surgeon;

(q) administration of veterinary medicines, in accordance with the label approved under the Agvet Code of Tasmania in relation to that medicine, by subcutaneous or intramuscular injection, oral administration (except oesophageal intubation) or application to any external body surface;

(r) anaesthetising and sedating of –

(i) fish of the class Osteichthyes; or

(ii) sharks, rays, lampreys or other cartilaginous fish of the classes Chondrichthyes and Agnatha;

(s) giving of advice on the nutrition and management of animals;

(t) foot trimming;

(u) ear tagging, ear marking or ear tattooing of any species;

(v) branding of any species;

(w) examination for pregnancy by the external application of ultrasound scanning in any species;

(x) artificial insemination, provided that the semen is introduced via the vagina and cervical canal.

[28] Government departments (re approval of joint projects): In the case that DPIWE or PWS has daily care of the animals, the signature of the person who will look after the animals must be obtained before this application is submitted. (See Section H: Declarations).

APPENDIX

Rat Monitoring Checklist 1: Pre-experiment

Look at the back of this sheet for intervention points, actions to be taken and emergency numbers

Project Title:							
AEC Project Approval number:							
Group/Animal ID :							
Procedure:							
Date							
Day							
Time							
Animal's weight (gr)							
Animal's weight change (%)							
➢ *Score the following by "**X**" if true, otherwise leave blank if no abnormalities are detected.* ➢ *If you want to add a comment, put a **NUMBER** and explain in the area at the back of this sheet.* ➢ *For the **Pain Sings**, indicate with "**X**" with its **reference letter** as shown at the back of this sheet.*							
Category 1							
Rough or ruffled fur, ungroomed							
Decreased activity							
Mild pain (see *ref list, turn page*)							
Aggressive when handled							
Category 2							
Moderate pain *(see ref list, turn page)*							
Vocalisation							
Extreme weight loss (>20%)							
Shallow or increased respiration							
Hunched posture							
Pale or cyanotic ears/nose/feet							
Dehydrated: sunken eyes, positive pinch test, square tail							
Poor body condition: vertebral spinous processes, scapulae, etc							
Category 3							
Severe pain *(see ref list, turn page)*							
Moribund							
Sings of blood							
Other?							
Signature (initials)							

Rat Monitoring Checklist 2: Post-experiment

Look at the back of this sheet for intervention points, actions to be taken and emergency numbers

Project Title:							
AEC Project Approval number:							
Group/Animal ID :							
Procedure:							
Date							
Day							
Time							
Animal's weight (gr)							
Animal's weight change (%)							
➢ *Score the following by "**X**" if true, otherwise leave blank if no abnormalities are detected.* ➢ *If you want to add a comment, put a **NUMBER** and explain in the area at the back of this sheet.* ➢ *For the **Pain Sings**, indicate with "**X**" with its **reference letter** as shown at the back of this sheet.*							
Category 1							
Rough or ruffled fur, ungroomed							
Decreased activity							
Mild pain (see *ref list, turn page*)							
Aggressive when handled							
Category 2							
Moderate pain *(see ref list, turn page)*							
Vocalisation							
Extreme weight loss (>20%)							
Shallow or increased respiration							
Hunched posture							
Pale or cyanotic ears/nose/feet							
Dehydrated: sunken eyes, positive pinch test, square tail							
Poor body condition: vertebral spinous processes, scapulae, etc							
Category 3							
Severe pain *(see ref list, turn page)*							
Moribund							
Sings of blood							
Other?							
Signature (initials)							

General Guidelines

If **ANY** of the above indications are marked with an X, then inform the Supervisor and apply the Actions To Be Taken according to the intervention points in the list below. If you have any General Comments, then add them below in the appropriate space provided. **SUPERVISORS CONTACT**: *Nikolas Dietis (office: 62261003, mobile: 0467981698) and Nuri Guven (office: 62261715, mobile: 0470380090).*

Pain References

Use the letters in the pain references below to accompany your X mark in the list above, where appropriate.

Sings of Mild Pain:	Sings of Moderate Pain:	Sings of Severe Pain:
A. Squinting eyes B. Porphyrin around eyes/nose C. Piloerection/rough coat D. Increased aggression E. Decreased exploratory behavior F. Vocalization when prodded G. Licking/scratching/self-trauma H. Guarding (protecting painful area) I. Reluctance to move J. Grinding of teeth (not attributed to pleasure) K. Reduced appetite	A. Inability to press paws/legs, B. Sings of inflammation in joints, C. Persistent vocalization, D. Staggering, E. Eyes closed F. Unresponsive G. Sunken/distended abdomen H. Hunched posture- Head tucked I. Abnormal gait (e.g.,ataxia, limping) J. Avoidance of contact with human K. Huddled facing corner in cage L. Tachycardia (faster than normal heart rate)	A. Writhe B. Twitch & fall C. Head pressing/bumping D. Labored breathing E. Curling up biting own feet (seen primarily with ureteral/bladder stones) F. Whimper (similar to dog whine but softer sounding heard in acute, severe pain)

Actions To Be Taken when an X is inserted in the Monitoring List above

If any of the below are followed, you need to complete and attach an Action Report documenting the steps taken.

Category 1 (Step 1-3 are mandatory)

Step 1: Schedule and execute a second screening within 4-5 hours.
Step 2: Include the animal in the scheduled experiment of the day.
Step 3: If monitoring is not improved after your second screening then inform your Supervisor.
Step 4: Depending on the stage of the experiments, some of the signs in this category might be treated as "expected" and no further actions will be needed (i.e. reduced activity in a depression study). Persistent or repeated unexpected Category 1 indications (1-2 days) will be treated as Category 2 indications by the Supervisor and actions will be taken accordingly.

Category 2 (Step 1-4 are mandatory)

Step 1: Schedule and execute a second screening within 4-5 hours and inform your Supervisor.
Step 2: Exclude animal from the scheduled experiment.
Step 3: If monitoring is improved, then treat as Category 1 and follow the necessary actions.
Step 4: If moderate pain, administer morphine 10mg/kg (sc) immediately.
Step 5: If other symptoms than pain, consult veterinarian . Depending on the behaviour recorded, persistent or repeated unexpected Category 2 indications will be treated as Category 3 indications by the Supervisor and actions will be taken accordingly.

Category 3 (All Steps are mandatory)

Step 1: Contact Supervisor and veterinarian.
Step 2: Exclude animal from the scheduled experiment.
Step 3: Schedule and execute euthanasia.
Step 4: Post mortem examination performed and complete an adverse event report.

Additional Comments

Appendix C. Approval from journals to reuse publications in this thesis

UK JOURNAL OF PHARMACEUTICAL AND BIOSCIENCES
Email: editor@ukjpb.com, Visit us at: www.ukjpb.com

UKJPB/2017/124 Date: 03/08/2017

TO WHOM SO EVER IT MAY CONCERN

This is to certify that Mr Alok Kumar Paul has published original work entitled **"Opioid receptor-dependent modulation of insulin-release in pancreatic beta-cells"** in UK Journal of Pharmaceutical and Biosciences Vol. 2(6), 2014.

As per information the published paper is a part of his PhD work. The publisher has no any objection if he incoporate the whole article in his PhD thesis.

We wish him success for his better future.

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INTRODUCTION